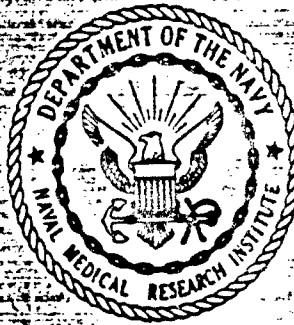20030131066

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# 1987 TOXIC HAZARDS RESEARCH UNIT ANNUAL REPORT

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ANNUAL REPORT FOR PERIOD COVERED OCTOBER 1986–OCTOBER 1987

HARRY G. ARMSTRONG AEROSPACE MEDICAL RESEARCH LABORATORY
HUMAN SYSTEMS DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

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This report has been prepared as a review of the activities of the Toxic Hazards Research Unit for the period of October 1986 through September 1987. Research activities focused on toxicity evaluations of aerospace and naval chemicals and materials to include hydraulic fluids, lubricating oils, groundwater contaminants, chemical defense simulants and binary agent components. Physiologically based pharmacokinetic/pharmacodynamic modeling has been developed and provides methods for simulating the toxicolgical effects of selected chemicals, including models for chemical carcinogenesis. The completion and utilization of the General Toxicology Laboratory within the THRU has resulted in the completion of an increased number of acute and repeated treatment studies, and additional subchronic studies are planned. *Cont'd pg 1*

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diisopropylfluorophosphate
halocarbon oil
hepatocytes
hydraulic fluid
inhalation
irritation
lactate dehydrogenase
lung
methylphosphonite,o-ethyl-o-(diisopropylaminoethyl)
mutagenicity
nephropathy
ortho-cresol
partition coefficient
pentanoic acid,2,3,4-trimethyl-1
perchloroethylene
peroxide
physiologically based pharmacokinetic model
phosphonite,o,o-diethylmethyl
quantitative structure activity relationship
respiratory epithelium
Salmonella
sensitization
simulation
sister chromatid exchange
toluene
transformation
trichloroacetic acid
trichloroethanol
trichloroethylene
trimethylpentane
unscheduled DNA synthesis

## PREFACE

The 24th annual report of the Toxic Hazards Research Unit (THRU) presents research and support activities performed by Northrop Services, Inc. - Environmental Sciences (NSI-ES) on behalf of the U.S. Air Force and the U.S. Navy under Contract Number F33615-85-C-0532. This document represents the second report under the current contract and describes the accomplishments of the THRU from October 1986 through October 1987.

Operation of the laboratory under this contract was initiated in January 1986 under Project 6302, "Occupational and Environmentai Toxic Hazards in Air Force Operations," Task 00, Toxic Hazards Research, Work Unit number 63020001. This research effort is cosponsored by the Naval Med cal Research and Development Command under the Naval Medical Research Institute, Toxicology Detachment, Navy Task Area number MF58524001, "Chemical Hazards/Exposure Limits."

Melvin E. Andersen, Ph.D., Acting Director of the Toxic Hazards Division, Harry G. Armstrong Aerospace Medical Research Laboratory, assumed the duties of the Technical Contract Monitor for this contract in May 1987.

Commander David A. Macys, MSC, USN, assumed direction of the U.S. Navy portion of this contract in August 1987.

The objectives of this research program are to identify and characterize the toxic effects of chemicals, compounds, and materials that are of operational interest and concern to the Air Force and Navy. This coordinated USAF-USN program generates scientific information from which simulation and predictive models, experimental design, and risk assessment criteria can be developed.

William E. Houston, Ph.D., served as Program Manager for NSI-ES and as the Director of the THRU. The preparation of this report represents significant contributions by the NSI-ES staff of the THRU at Wright-Patterson Air Force Base, OH, and the NSI-ES staff at Research Triangle Park, NC.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AALAS | - | American Association for Laboratory Animal Science |
| AAMRL | - | Armstrong Aerospace Medical Research Laboratory |
| ACA | - | Automated Clinical Analyzer |
| ACSL | - | Advanced Continuous Simulation Language |
| BALB/c | - | Strain of mouse the cells of which are used for transformation assays |
| B(a)P | - | Benzo(a)pyrene |
| Be | - | Beryllium |
| BeO | - | Berryllium oxide |
| BSA | - | Bovine serum albumin |
| BuDr | - | Bromodeoxyuridine |
| BUN | - | Blood urea nitrogen |
| CAb | - | Chromosome aberration |
| CAS | - | Chemical Abstracts Services |
| CFE | - | Colony forming efficiency |
| CHO | - | Chinese hamster ovary |
| COBAS | - | Clinical Biological Analyzer Systems |
| CPFB | - | Chloropentafluorobenzene |
| CPK | - | Creatinine phosphokinase |
| CPR | - | Cardiopulmonary resuscitation |
| CTFE | - | Chlorotrifluoroethylene |
| CTP | - | Cyclotriphosphazene |
| CW | - | Chemical warfare |
| DFP | - | Diisopropylfluorophosphate |
| DMBA | - | 7,12-dimethylbenz(a)anthracene |
| DMEM | - | Dulbecco's Modified Eagles Medium |

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| | | |
|---|---|---|
| DMMP | - | Dimethylmethylphosphonate |
| DMSO | - | Dimethyl sulfoxide |
| ECD | - | Electron capture detector |
| EDTA | - | Ethylene-diaminetetraacetic acid |
| EMEM | - | Eagles Minimum Essential Medium |
| EPA | - | Environmental Protection Agency |
| FBS | - | Fetal bovine serum |
| FID | - | Flame ionization detector |
| $g$ | - | Gravity |
| GC | - | Gas chromatography |
| GGPT | - | Gamma gutamyl transpeptadase |
| GLP | - | Good laboratory practices |
| HBSS | - | Hank's balanced salt solution |
| HBSSA | - | A modified Hank's balanced salt solution for washing primary hepatocytes |
| HEPA | - | High efficiency particulate air (or absolute filter) |
| HGPRT | - | Hypoxanthine-guanine phosphoribosyl transferase |
| HP | | Hewlett-Packard |
| HPLC | - | High pressure liquid chromatography |
| IRP | - | Installation Restoration Program |
| IVPL | - | Isolated ventilated perfused lung |
| JMEM | - | Joklik's Modified Eagle Medium |
| KB | - | Diisopropylaminoethanol |
| LDH | - | Lactate dehydrogenase |
| LT | - | Bis(diisopropylaminoethyl)methylphosphonite |
| MCH | - | Mean corpuscular hemoglobin |
| MCHC | - | Mean corpuscular hemoglobin concentration |
| MCV | - | Mean corpuscular volume |

| | | |
|---|---|---|
| MNNG | - | N-methyl-N'-nitro-N-nitrosoguanidine |
| MS | - | Mass spectrometry |
| MSDS | - | Manufacturer's safety data sheet |
| NADP | - | Nicotinamide adenine dinucleotide phosphate |
| NIEHS | - | National Institute of Environmental Health Sciences |
| NIOSH | - | National Institute for Occupational Safety and Health |
| NMRI/TD | - | Naval Medical Research Institute/Detachment (Toxicology) |
| 4NQO | - | 4-Nitro-quinoline-1-oxide |
| NSI-ES | - | Northrop Services, Inc. – Environmental Sciences |
| NTP | - | National Toxicology Program |
| OP | - | Organophosphate |
| PB-PK | - | Physiologically-based pharmacokinetic |
| PBS | - | Phosphate buffered saline |
| PCE | - | Perchloroethylene |
| PCM | - | Pharmacodynamic cancer model |
| QSAR | - | Quantitative structural activity relationships |
| QL | - | o-Ethyl-o'-(diisopropylaminoethyl)methylphosphonite |
| RBC | - | Red blood cell |
| RCE | - | Relative cloning efficiency |
| RDW | - | Red cell distribution width |
| REM | - | Rocket motor exhaust materials |
| RH | - | Relative humidity |
| RTE | - | Rat tracheal epithelial |
| RTP | - | Research Triangle Park, NC |
| SAS | - | Statistical Analysis System |
| SCE | - | Sister chromatid exchange |
| SEM | - | Standard error of the mean |

| | | |
|---|---|---|
| SGOT | - | Serum glutamic-oxalacetic transaminase |
| SSMS | - | Spark source mass spectroscopy |
| TCA | - | Trichloroacetic acid |
| TCE | - | Trichloroethylene |
| TCOH | - | Trichloroethanol |
| TEP | - | Triethylphosphite |
| TFE | - | Trifluoroacetylacetone |
| 6-TG | - | 6-Thioguanine |
| TH | - | Toxic Hazards Division |
| THRU | - | Toxic Hazards Research Unit |
| TMP | - | 2,3,4-Trimethylpentane |
| TMPA | - | 2,3,4-Trimethyl-1-pentanoic acid |
| TR | - | o,o'-Diethylmethylphosphonite |
| TRO | - | Diethylmethylphosphonate |
| UDS | - | Unscheduled DNA synthesis |
| USAF | - | United States Air Force |
| WBC | - | White blood cell |
| WPAFB | - | Wright-Patterson Air Force Base |
| YL | - | Ethyl methyl phosphonate |

# SECTION 1

## SUMMARY

Research activities and support operations of the Toxic Hazards Research Unit (THRU) are conducted as a continuing program independent of contract years. Technical directives (TDs) generated by the Air Force and Navy constitute distinct research efforts that may transcend annual reporting periods. Research activities focused on toxicological evaluations of aerospace and naval chemicals and materials, including hydraulic fluids, fuels, lubricating oils, ground water contaminants, chemical defense simulants, and binary agent components. The research program this year included expansion of efforts to develop physiologically-based pharmacokinetic (PBPK) modeling to provide a better understanding and predictive capability of the distribution and metabolism of chemicals in biological systems. This new research effort, the Biological Simulation Program, conducts simulation modeling in support of testing and research in the THRU to guide experimental design, to aid in interpretation of experimental data, and to assist in extrapolating animal data to man and in assessment of risk.

Among the major accomplishments this year was the completion of the General Toxicology Laboratory. This effort required assistance from and cooperation with USAF personnel in installing and expanding the electrical capability of the research space, installation and operation of six new THRU inhalation chambers, and installation and operation of isolation rooms. Significant effort was expended in modifying existing chamber air supply and exhaust systems to bring these systems into operation. The addition of these six chambers increased the inhalation capabilities of the THRU, and the isolation rooms provided facilities for dermal and oral dosing as well as animal observation.

During the second year of operation, research focused on acute to subchronic toxicity studies. One of these studies, the first 90-day investigation in the new THRU inhalation chambers, is being conducted on chlorotrifluoroethylene (CTFE) oligomers, a nonflammable, halogen-saturated lubricating oil which is noncorrosive and provides good lubricity. Previous studies indicated a low degree of toxicity and no organophosphate-type pathogenicity, whereas the ongoing studies will investigate the irritation and sensitization potential associated with repeated or continuous exposure to CTFE.

The Air Force is conducting a large-scale program to identify, characterize, and manage hazardous waste sites at Air Force installations worldwide. Although long-term monitoring, risk assessment, and abatement efforts are included in the overall management program, many installations have short-term, site-specific problems. The THRU's assistance to the short-term problem at WPAFB includes both acute (oral toxicity, skin and eye irritation, and sensitization) and

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repeated treatment (14-day oral and dermal) studies of environmental samples. The first three leachate samples have not exhibited any toxicity or potential for sensitization. To study further the dermal absorption of volatile organic chemicals detected in contaminated ground water, a dermal uptake model is being developed at the THRU. Dermal absorption kinetics of three such chemicals, trichloroethylene (TCE), perchloroethylene, and toluene, have been determined at NSI-ES in Research Triangle Park (RTP) and will be used to refine this model. Results from studies with an isolated ventilated perfused lung (IVPL) preparation developed this year at the THRU indicated that the rat lung accounts for only about 0.0001 of the whole body's ability to metabolize TCE. These studies were performed using both blood and artificial perfusate to maintain the lung and by exposing the lung to various concentrations of TCE. Future studies will be conducted to compare results using blood and artificial perfusates in the IVPL.

Research on fuels or fuel additives of interest to the Air Force are in progress at the THRU, at NSI-ES in RTP, and on subcontract. Studies at the THRU include physical characterization and *in vivo* clearance of fuel exhausts which contain beryllium oxide (BeO). Exhaust material from rocket propellents are being compared with beryllium oxide formed at low- (less than 500°C) and high- (above 1600°C) firing conditions. Chemical analyses were performed on several samples of rocket exhaust. Little difference was seen in these analyses, and the samples were pooled. This pooled sample was ground to smaller particle size specifications for use in solubility studies and nose-only inhalation studies to compare it with the high- and low-fired BeO compounds. These studies are aimed at providing information on the deposition and clearance of all three materials. Using the rocket exhaust samples ground to inhalation specifications at the THRU, the NSI-ES *In Vitro* Toxicology Laboratory at RTP found indications of toxicity to rat tracheal epithelial cells. The low-fired BeO and rocket exhaust samples appear to be more toxic than the high-fired compound. Transformation data from *in vitro* studies are now being collected. Another fuel study at the THRU is focused on determining liver toxicity of 2,3,4-trimethylpentane (TMP) in rat hepatocyte suspension cultures. TMP, a hydrocarbon propellent, was shown by lactate dehydrogenase (LDH) leakage and viability studies to produce *in vitro* hepatotoxicity at concentrations of $1.46 \times 10^{-2}$ M. Finally, on subcontract, the THRU identified several metabolites of alkyl substituted cyclohexane hydrocarbon fuels that are associated with nephropathy in rats. Eleven of these compounds, which are metabolites of *t*-butylcyclohexane, have been identified and synthesized for use in prospective *in vivo* nephrotoxic potential studies by the Air Force.

A biologically-based computer simulation model for chemical carcinogenesis has been developed among scientists at the THRU, the Air Force, and Dow Chemical Company in which mammalian physiology and carcinogen metabolism have been mathematically described. The biochemical mechanisms for DNA-damaging agents, promoters, and cytotoxicants are elaborated in

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which the target tissue concentration of parent compound or metabolite is linked to tumor development, and tissue dose of toxicant can be derived from the applied dose. This model can be used to simulate the effects of lifetime exposures to maximum tolerated doses as well as exposure to expected environmental doses. Validation studies for cytotoxicants are currently in progress with chloroform. These models, appropriately validated, may provide powerful tools for developing realistic risk estimates for carcinogenic compounds. In support of the PB-PK modeling effort, the THRU, on subcontract to one of the local universities, is developing quantitative structure activity relationships (QSAR) for several chemicals. QSAR employs theoretical methods to estimate the role of molecular structural features in the metabolism of the chemicals. Optimized orbital geometries for 25 parent compounds have been obtained and compared with experimental gas phase structures. These theoretical and experimental results have shown good correlation for 18 of the compounds.

A PB-PK and pharmacodynamic model is being developed to simulate the time course of organophosphates (OP) in the blood and brain, and to correlate these measurements with inhibition of OP-susceptible esterases (acetylcholinesterase and carboxylesterase). A representative OP, diisopropylfluorophosphate was the initial compound studied. The $LD_{50}$ for this compound (1.75 mg/kg) in rats was established to provide information for a nonlethal exposure level to be used in validating the model. In addition, partition coefficients required for the model have been determined for several tissues.

Chloropentafluorobenzene (CPFB), an Air Force candidate chemical agent simulant, was found to have potential genotoxic activity as measured by a series of in vitro genotoxicity tests. Suggestion of cytogenic abnormalities was found in the sister chromatid exchange (SCE) assay, and marginally positive results were evident in the chromosome aberration assay and in the BALB/c-3T3 transformation assay. A PB-PK model incorporating cardiac output, ventilation rate, and breath concentrations of CPFB has been developed in which tissue concentrations of CPFB can be predicted by measuring expired air from rats receiving inhalation exposure doses from 300 to 1200 ppm CPFB vapor. In an initial effort to validate this model, measured concentrations of expired CPFB from rats corresponded well with model simulations of the same exposures. This model may be useful for evaluation of breath monitoring with expired toxicants as well as with uptake simulants.

A cooperative Air Force/Army research effort for the evaluation of the potential toxicity of several by-products associated with the manufacture of o-ethyl-o'-(diisopropylaminoethyl) methylphosphonite (QL), a binary component of one of the chemical agents, is in progress. The THRU designed and developed an exposure system that controls the rapid chemical reactivity of o,o'-diethylmethylphosphonite (TR), a product of a disproportionation reaction of QL catalyzed by water. This system will be used to expose animals to a chemically stable atmosphere of TR hydrolysis

products, as well as provide a basis for quantitation of the starting TR component. Acute inhalation studies are scheduled to begin in the first quarter of the third contract year.

The THRU has completed dermal toxicity studies on an "activated peroxide" compound, which the Navy is considering as a decontaminant for skin exposed to chemical warfare agents. This decontaminant was found to be safe for human skin contact (by the NIOSH Skin Test Rating System) if allowed to remain on the unoccluded skin of test animals 'or 30 min or less, and followed by a detergent or soap and water wash. Occlusion of the skin, however, enhanced the irritational effects of the decontaminant.

A number of hydraulic fluids and lubricating oils were tested for toxicity for the Navy. Cyclotriphosphazene (CTP) hydraulic fluid exhibited no acute oral or dermal toxicity, skin or eye irritation, or skin sensitization. Twenty-one day repeated inhalation and dermal studies have just been completed, and evaluation of the data and processing of tissues for histopathology studies are in progress. Rats treated orally with Halocarbon 27-S lubricating oil, a polymer of CTFE, demonstrated a decrease in water consumption and daily urine output. Significant increases in the fluoride content of the urine were observed, neurotoxic symptoms were found in female rats (male rats were asymptomatic), and significant increases in liver and kidney weights were seen in both sexes. Acute inhalation toxicity studies with Bel-Ray Syncom 1400, a synthetic lubricant, did not disclose toxic effects, nor were gross lesions reported upon necropsy. Chemical analysis of a hydraulic fluid mixture involved in a shipboard exposure incident revealed similar gas chromatography (GC) elution patterns to known neurotoxic phosphate triester materials containing o-cresol previously studied at the THRU. Based upon these data, neurotoxicity ar.'mal studies are scheduled early in 1988.

The completion and operation of the General Toxicology Laboratory this year represents a major step forward in the THRU's ability to perform acute to subchronic inhalation studies. Six inhalation chambers, designed and developed by THRU scientists, provide an estimated fivefold increase in our inhalation capabilities, and two large isolation rooms provide new oral and dermal dosing facilities and animal observation rooms. Isolation rooms being installed within the pharmacokinetic laboratory will add secondary containment necessary for research activities in the Biological Simulation Program.

# SECTION 2

# INTRODUCTION

## 2.1 FACILITIES AND PROGRAM OBJECTIVES

Northrop Services, Inc. – Environmental Sciences (NSI-ES), during its second year of operating the Toxic Hazards Research Unit (THRU), completed major upgrades in the toxicology testing equipment and facilities. THRU scientists designed new inhalation chambers, which are now in use. A General Toxicology Laboratory, which is now operational, includes the new chambers designed for acute to subchronic inhalation toxicology studies and isolation rooms in which dermal and oral toxicology studies are being conducted. These rooms permit the isolation of animals treated with volatile or toxic materials and the individual housing of multiple animal species to meet the requirements of the American Association for Accreditation of Laboratory Animal Care. An exposure room has also been constructed in the laboratory conducting pharmacokinetic and pharmacodynamic studies. This room will provide the secondary containment for studies involving highly toxic agents, such as organophosphate chemicals; carcinogenic compounds; and chemicals with unknown toxicity, such as candidate simulant agents.

The research efforts have been considerably expanded and consist of three mutually cooperative research programs. Computer simulation modeling is conducted by the newly formed *Biological Simulation Program* to support testing and research in the THRU. These models are used predictively to guide experimental design, aid in the interpretation of experimental data, and for extrapolation of research information to assess risk. These simulation efforts rely upon mathematical descriptions of the chemical, biochemical, and physiologic processes underlying acute and chronic toxicity. The *Engineering and Chemistry Program* maintains and modifies the research and testing equipment and facilities, develops new and unique approaches to vapor and aerosol generation systems for inhalation studies involving chemically reactive substances, and provides analytical chemistry methodology necessary to provide data to the modeling effort. The *Toxicology Program* conducts inhalation, dermal, irritation, and neurotoxicity studies to provide information to the Air Force and Navy concerning the toxic effects of comp unds developed for use in the Armed Forces. This information provides validation of predictive models developed at the THRU. This program also includes the necessary animal resources, necropsy, and histopathology support for ongoing studies across all programs. An *in vitro* toxicology section provides cell culture capabilities and also conducts studies on metabolism of selected xenobiotics by isolated hepatocytes and kidney tubule cells.

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The THRU in-house program is supported in part by subcontracted research with local scientists and university affiliations. One of these research efforts employs quantitative structural activity relationships (QSAR) methods to estimate the relationship of molecular structure to metabolism of compounds, while another effort has identified and synthesized metabolites of cyclohexane hydrocarbon fuels associated with nephropathy in rats. The scientific capabilities of the THRU are expanded through research efforts conducted at NSI-ES, Research Triangle Park (RTP), NC. *In Vitro* genotoxicity studies were conducted on rocket fuels containing beryllium and chloropentafluorobenzene (CPFB), a chemical simulant. Studies were also performed to measure the dermal absorption kinetics of selected chemicals frequently found in groundwater.

The NSI-ES Scientific Advisory Board and selected consultants held a program review during the last quarter of fiscal year 1987. In this initial review of the NSI-ES research and support program, the evaluating panel recognized a maximum utilization of existing facilities and reflected positively on the facilities that were upgraded to conduct necessary studies. Recommendations from this panel will be taken into consideration when planning future studies with Air Force and Navy managers.

## 2.2   TOXICOLOGY CONFERENCE

The 1987 Conference on Toxicology, the 17th in this series of forums, brought together nationally and internationally recognized authorities under the theme *Quantitative Toxicology*. Papers were presented in the fields of occupational toxicology, pharmacokinetics and experimental design, bioeffects modeling, the application of emerging techniques in predictive toxicology, and quantitative methods in behavioral toxicology. Computer demonstrations presented programs that are used both for predictive and quantitative purposes in toxicology. This session clearly illustrated how the power of the computer will be utilized today and in the future to address selected toxicologic end points. A poster session was held to address additional topics concerning quantitative methods in toxicology. This conference is jointly sponsored by the Toxic Hazards Division of the Armstrong Aerospace Medical Research Laboratory (AAMRL/TH) and the Toxicology Detachment of the Naval Medical Research Institute (NMRI/TD).

## TOXICOLOGY EVALUATIONS OF AEROSPACE CHEMICALS AND MATERIALS

### 3.1 SUBCHRONIC STUDIES OF CHLOROTRIFLUOROETHYLENE

E. Kinkead, B. Culpepper, S. Henry,
E. Kimmel, V. Harris, and R. Kutzman

### INTRODUCTION

Chlorotrifluoroethylene (CTFE) oligomer is an inert, nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil. It is noncorrosive, has high thermal stability, good lubricity, and high dielectic strength. Recent dermal and inhalation studies indicate that CTFE has a low degree of toxicity. There were no deaths among male and female rabbits dermally exposed to 2 g CTFE/kg body weight (Gargus, 1983). There were no deaths among male and female rats exposed for 4 h to atmospheres containing saturated-vapor concentrations of CTFE (Coate, 1984; Kinkead et al., 1987).

CTFE was readily absorbed and converted to free fluoride following oral and inhalation exposures. Plasma and urine fluoride levels remained elevated for more than one week following oral exposure and for at least 24 h following inhalation exposure. However, CTFE absorption was not evident following dermal exposure (Kinkead et al., 1987). Histopathologic examination of nerve tissue from hens dosed with CTFE showed no lesions consistent with organophosphate toxicity. The acute toxicity of CTFE compares favorably with other hydraulic fluids tested in this laboratory. However, neither the irritation and sensitization potential nor the effects associated with repeated or continuous exposure to CTFE have been investigated.

The subchronic inhalation studies reported here will provide information on health hazards likely to arise from repeated exposures by the inhalation route over a limited time. It will provide information on target organs and can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

### MATERIALS AND METHODS

#### Animals

Upon receipt from Charles Rivers Breeding Labs, Kingston, NY, 40 male and 40 female Fischer 344 rats, 9-11 weeks of age, were quality control tested and found to be in acceptable health. The animals were randomized and group housed (two to three per cage) in clear plastic cages with wood chip bedding prior to the study. The rats were housed individually and assigned to specific exposure cage locations during the study. The exposure cages were rotated clockwise (moving one position)

within the THRU inhalation chambers each exposure day. Water and feed (Purina Formulab #5008) were available *ad libitum* except during the inhalation exposure period and when the rats were fasted for 10 h prior to sacrifice. The light/dark cycle was set at 12-h intervals.

### Test Agent

This formulation of the CTFE oligomer contains 1.0% (vol/vol) of a rust inhibitor additive, neutral barium dinonylnaphthalene sulfonate. A second additive is 0.05% (vol/vol) of a proprietary anti-wear compound (composition unknown).

Chemically, halocarbon oils are saturated, low molecular weight polymers of chlorotri-fluoroethylene that have the general formula $(CF_2CFCl)_n$. They are made using a controlled polymer-ization technique and are stable, the terminal groups being completely halogenated and inert. The product is then separated by vacuum distillation into various fractions, from light oils to waxes.

### Test Agent Quality Control

The batch lot of CTFE used in this study was analyzed by gas chromatography using electron capture detection (GC-ECD). The composition was verified by gas chromatography-mass spectrometry (GC-MS). A high pressure liquid chromatography (HPLC) method was developed to ensure that high molecular weight oligomers would be detected if present.

### INHALATION TOXICITY

### Generation and Analysis

Exposure atmospheres were generated by aerosolization using Collison (BGI, Inc., Waltham, MA) compressed air nebulizers. A single jet nebulizer was used to generate the 0.25- and 0.50-mg CTFE/L concentrations, whereas a three-jet nebulizer was required to generate the 1.0-mg CTFE/L atmosphere. Chamber atmospheres produced by this generation system contained both vapor and aerosol fractions at a 90:10 proportion. Aerosol concentrations within the exposure chambers were determined by gravimetric analysis of aerosol collected on glass-fiber filter media. The size distribution of the aerosols was determined by a Lovelace Multijet Cascade Impactor (Intox Products, Albuquerque, NM). The chamber vapor concentrations were monitored by Miran (Foxboro, MA) infrared analyzers at a wavelength of 1200 cm$^{-1}$.

### Exposure Regimen

Ten male and ten female F-344 rats, age 9-11 weeks, were placed in each of four 690-L Toxic Hazards Research Unit (THRU) inhalation chambers and exposed for 6 h/day, 5 days/week, for 13 weeks (65 exposures over a 90-day test period) to air only, 0.25 mg CTFE/L, 0.50 mg CTFE/L, and 1.00 mg CTFE/L, respectively.

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Records were maintained for body weights (1 day pre-exposure, weekly during the first four weeks, then biweekly thereafter), signs of toxicity, and mortality. At sacrifice (study is currently in progress), gross pathology will be performed and tissues (Table 3.1-1) harvested for histopathologic examination. Additionally, blood will be drawn for hematology (Table 3.1-2) and clinical chemistry (Table 3.1-3) assays. Wet tissue weights will be determined on adrenals, brain, heart, kidneys, liver, lungs, ovaries (females), spleen, testes (males), and thymus.

TABLE 3.1-1. TISSUES HARVESTED FOR HISTOPATHOLOGIC EXAMINATION

| | |
|---|---|
| Gross lesions | Thymus |
| Brain | Kidneys |
| Lungs | Adrenals |
| Trachea | Pancreas |
| Heart | Ovaries/testes |
| Liver | Nasal turbinates |
| Spleen | Uterus (females) |
| Duodenum | Esophagus |
| Jejunum | Stomach |
| Ileum | Colon |
| Urinary bladder | Rectum |
| Mandibular lymph nodes | Sternum |
| Mesenteric lymph nodes | Sciatic nerve |
| Teeth (incisors) | Skeletal muscle |
| Bone (femurs, including stifle) | |

TABLE 3.1-2. ASSAYS PERFORMED ON WHOLE BLOOD

| |
|---|
| Hematocrit |
| Hemoglobin |
| Red blood cell count |
| Total and differential leucocyte count |

TABLE 3.1-3. SERUM CHEMISTRIES PERFORMED

| | |
|---|---|
| Creatinine | Alkaline phosphatase |
| Chloride | Blood urea nitrogen |
| Calcium | Serum glutamic-pyruvic transaminase |
| Phosphorus | Serum glutamic-oxalacetic transaminase |
| Total protein | |

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## Statistical Analysis

Body weight means and associated standard errors (SEM) will be calculated according to Dixon (1985). Comparison of body weights will be performed using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A two-factorial analysis of variance with multivariate comparisons will be used to analyze the hematology, clinical chemistry, and organ weight data. The histopathology data will be analyzed using one of the following nonparametric tests: Fisher's Exact Test or, if not valid, Yates' Corrected Chi-square (Zar, 1974). Probability of 0.05 will infer a significant change from controls.

## RESULTS

This study is currently in progress.

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STUDIES ON INSTALLATION RESTORATION CHEMICALS

## 4.1 DEVELOPMENT OF AN ISOLATED VENTILATED PERFUSED LUNG PREPARATION

A. Vinegar and D. Brown

### INTRODUCTION

The nonrespiratory metabolic functions of the lung have received increased attention in recent years. Knowledge of these functions and interest in the fate of xenobiotic substances have resulted in increased use of the isolated ventilated perfused lung (IVPL) preparation as an attempt to localize the contribution of the lungs to the metabolism of these substances. In some cases, the lung itself may be the target for toxic effects of the parent compound and/or its metabolites.

Recent development of the IVPL has focused heavily on mechanical aspects of the preparation. If a physiologically viable isolated lung is to be maintained for any reasonable length of time, these mechanical refinements are necessary. The results of the early development period are presented herein. The studies summarized are those conducted with whole blood perfusate circulated through the IVPL apparatus without a lung in place.

### EXPERIMENTAL PARAMETERS INVESTIGATED

#### Perfusate Acquisition

The manner in which blood is acquired for perfusion may be critical to the success of the preparation. The blood must be obtained in as much a state of physiologic normality as possible, unhemolyzed by the donation process, and with clotting inhibited by use of an anticoagulant.

Some delay in the onset of hemorrhagic shock may be realized by maintaining the circulating volume of the donor with a physiologic solution during the donation process, thereby maintaining tissue perfusion and oxygenation. In the perfusate studies for the IVPL, F-344 male rats (320-400 g) were anesthetized with 3.5% Halothane vapor and laparotomized to expose the abdominal aorta. The aorta was cannulated with a small diameter polyethylene tube, into which 2.0 mL of rat Ringer's solution containing 75 USP units of sodium heparin was injected. This volume corresponds to approximately 10% of the circulating blood volume of the subject.

The rat Ringer's/heparin solution was permitted to circulate in the vasculature of the donor rats for three to five minutes. At the end of this time, the cannula was connected to a heparinized syringe into which the donor was allowed to bleed. In this manner, blood was obtained without

using negative pressure, which can rupture cells and alter the chemistry of the perfusate. Some experimentation was required to arrive at the proper dose of heparin to prevent coagulation and also prevent cell damage due to over-heparinization of the blood. Typically, 9 to 11 mL of blood were obtained from each donor rat before cessation of useful cardiac activity. For each perfusate experiment, three donor rats were utilized, resulting in the collection, into a single siliconized glass flask, of approximately 30 mL of fresh blood. A sample of the pooled blood was removed from this flask for baseline (t = 0) blood cell and chemistry analyses.

### Pooling of Donor Blood

Some concern over the possibility of agglutination induced by pooling of blood acquired from multiple donors was addressed early in this research. All donor blood was obtained from the same strain of F-344 male rats; microscopic examination of the pooled, heparinized whole blood of three donor rats failed to demonstrate evidence of agglutination or microaggregation 1 h after pooling.

### Studies of the Circulating Perfusate

Twenty-five milliliters of the fresh donor blood were instilled into the blood reservoir of the IVPL apparatus. The jacketed reservoir of the apparatus maintained the circulating blood at 37°C. An anhemolytic, adjustable occlusion, peristaltic blood pump was designed and constructed specifically for the low priming volume associated with the IVPL. The pump circulated the perfusate, with minimal turbulence, from the reservoir into a closed loop of Silastic tubing in the artificial "thorax" and then back to the reservoir, through siliconized glass tubes. The closed loop will be replaced by the actual lung in later studies. In all perfusate studies, the pump was maintained at a "just occlusive" setting such that pump speed in revolutions per minute (rpm) and its output were linearly related, but hemolysis was minimized. Pump speed was always maintained at 22 rpm, providing an output of 60 mL/min. At this pump speed, a volume equal to that of the entire initial perfusate volume recirculated every 24 s. These conditions approximated the cardiac output of a 350-g rat.

Two different analysis schemes were developed for the perfusate, taking into consideration sample size requirements, heparin compatibility, and relevance as indicators of perfusate composition and osmolarity.

### Blood Cell Analysis

At the beginning and end of each perfusate experiment, and at intermediate points for some of the experiments, blood was removed for analysis by a Coulter automated blood cell counter. This device segregates the formed elements of the blood according to size distribution and counts the

number of elements within a specified range of size for a given volume of blood. Results are summarized in Table 4.1-1.

**TABLE 4.1-1. WHOLE BLOOD PERFUSATE HEMATOLOGY AND CHEMISTRY AT SELECTED TIMES DURING TEST RUNS PERFORMED IN THE IVPL APPARATUS WITHOUT A LUNG IN PLACE**

**Blood Cell Composition (N = 6)**

|  | t = 0 | t = 3 h |
|---|---|---|
| White blood cells | 2.3 (2.0 – 2.5)[a] x $10^3$/mL | 2.4 (2.0 – 2.8) x $10^3$/mL |
| Red blood cells | 7.30 (7.04 – 7.54) x $10^6$/mL | 7.23 (6.89 – 7.50) x $10^6$/mL |
| Hemoglobin (total) | 12.6 (12.1 – 13.1) g/dL | 12.3 (11.5 – 12.8) g/dL |
| Hematocrit | 35.9 (34.2 – 37.2)% | 35.2 (33.6 – 37.1)% |

**Chemistry Analysis[b] – Series A (N = 3)**

|  | t = 0 | t = 1 | t = 2 | t = 3 h |
|---|---|---|---|---|
| Glucose (mg/dL) | 250 (224 – 288) | 176 (150 – 202)[c] | 174 (99 – 259) | 155 (100 – 229) |
| Total protein (g/dL) | 4.97 (4.88 – 5.07) | 4.88 (4.68 – 5.09) | 4.65 (4.42 – 4.82) | 4.62 (4.50 – 4.75) |
| Calcium (mg/dL) | 10.0 (8.8 – 12.0) | 9.3 (8.7 – 9.6) | 10.2 (8.3 – 12.5) | 10.1 (8.8 – 10.8) |
| Cholesterol (mg/dL) | 41 (40 – 42)[c] | 46 (42 – 48) | 54 (53 – 56) | 57 (50 – 63) |
| Chloride (mg/dL) | 112 (108 – 116) | 112 (108 – 115) | 115 (114 – 115) | 118 (117 – 119) |
| Phosphorus (mg/dL) | 6.17 (4.77 – 7.70) | 5.97 (5.06 – 7.13) | 6.46 (5.34 – 7.50) | 6.64 (5.22 – 7.75) |
| Magnesium (mg/dL) | 1.4 (1.3 – 1.5)[c] | 1.4 (1.3 – 1.6) | 1.3 (1.2 – 1.4) | 1.3 (1.2 – 1.4) |

**Chemistry Analysis[b] – Series B (N = 3)**

|  | t = 0 | t = 1.5 | t = 3 h |
|---|---|---|---|
| Blood urea nitrogen (mg/dL) | 17.7 (15.5 – 19.0) | 17.1 (14.5 – 18.7) | 17.8 (16.2 – 19.0) |
| Creatinine (mg/dL) | 0.6 (0.5 – 0.6) | 0.7 (0.5 – 0.8) | 0.6 (0.6 – 0.7) |
| Potassium (mmol/L) | 4.07 (3.84 – 4.30)[c] | 6.09 (5.60 – 6.57)[c] | 7.44 (6.90 – 7.97)[c] |
| Carbon dioxide (mmol/L) | 22.2 (21.6 – 22.7) | 12.4 (10.7 – 13.8) | 9.5 (4.3 – 15.1) |
| Lactic acid (mmol/L) | 3.8 (3.6 – 4.2) | 9.7 (7.8 – 12.5) | 11.7 (8.4 – 14.1) |

[a] Numbers in parentheses represent the range of values.
[b] There are two sets of data under this heading because of different requirements for blood anticoagulant.
[c] N = 2.

## Hemolysis

In the six perfusion test runs that are reported here, the mean baseline hematocrit was 35.9%. After 3 h of recirculating in the apparatus, the mean hematocrit of the perfusate for the six runs was 35.2%, reflecting a decrease of 0.6%. Similarly, the mean starting red cell count for the six runs was 7.30 x $10^6$, and at t = 3 h was 7.23 x $10^6$, reflecting a mean decrease in perfusate red-cell population of slightly less than 1% of starting values.

### Baseline Carbon Dioxide Concentration

Carbon dioxide concentration of the perfusate consistently fell during recirculation through the apparatus. This decrease indicates that exposure of the perfusate to air is sufficient to allow off-gassing. Mean starting $CO_2$ concentration, in the three runs where it was measured, was 22.2 mmol/L. After 3 h of recirculation, mean $CO_2$ concentration was 9.5 mmol/L. These perfusate measurements of $CO_2$ will permit more accurate interpretation of blood gas data derived from preparations with lungs in place.

### Glucose and Lactic Acid

This study has clarified some of the baseline parameters of metabolism of the living perfusate, (specifically glucose consumption and lactic acid production) as it recirculates at physiologic temperature within the IVPL apparatus. Measurements of glucose and lactic acid concentrations during experiments with a lung in place can thus be corrected for perfusate metabolism.

In three perfusate test runs, the mean glucose concentration of the perfusate was 250 mg/dL at the start of circulation in the IVPL apparatus (after donation). After 3 h of circulation, the mean glucose concentration was 155 mg/dL, or 60.8% of the starting concentration. Initial lactic acid concentration in three perfusate samples averaged 3.8 mmol/L; at the end of 3 h of recirculation, this average concentration tripled to 11.7 mmol/L.

### Sodium and Potassium

Data for sodium and potassium concentration of the perfusate are not complete now due to a series of malfunctions of the Dupont automated clinical analyzer in the AAMRL/TH Clinical Pathology Laboratory. What potassium data are available indicate a marked increase in potassium concentration at the end of 3 h of recirculation. No sodium data are available. Such data, when available, will be useful in assessing the role of the sodium ion in the development of pulmonary edema and rising perfusion pressures commonly seen in IVPL preparations.

### CONCLUSIONS

The parameters examined in the described circulating perfusate studies will serve as background information to aid in the interpretation of experiments using blood as the perfusate in the IVPL preparation. Future development of the IVPL preparation will involve documenting the use of an artificial perfusate to avoid the problems associated with collecting and pooling blood from multiple animals to support one lung preparation.

## 4.2 METABOLISM OF TRICHOROETHYLENE IN THE ISOLATED VENTILATED PERFUSED LUNG

A. Vinegar, R. Conolly, K. Auten, and D. Cook

### INTRODUCTION

In conjunction with the development of the physical apparatus for the isolated ventilated perfused lung (IVPL), work has progressed on the development of a model of the apparatus. The model written in Advanced Continuous Simulation Language (ACSL) helps in conducting experiments using the IVPL preparation. The specific code used at present follows the metabolism by the lung of trichloroethylene (TCE) to trichloroacetic acid (TCA) and trichloroethanol (TCOH). The fraction of whole body metabolism contributed by the lung is based on fitting data from the paper by Dalbey and Bingham (1978) to the model. Simulations are being used to select the needed sensitivity of the analytical techniques for TCA and TCOH and the sampling intervals of the blood in the IVPL preparation. TCE is of interest to the Air Force as it is one of the chemicals of concern in the Installation Restoration Program.

### MATERIALS AND METHODS

The IVPL apparatus is similar to that described by Dalbey and Bingham (1978) except for the following differences. The pump being used can be run in a nonoccluding mode to reduce the onset of hemolysis. The blood reservoir is closed to the outside atmosphere preventing the loss of volatile compounds from the system. All airspaces within the system are accounted for in the model. Techniques for analysis of TCE, TCA, and TCOH also follow Dalbey and Bingham (1978).

Several preliminary runs of the IVPL were conducted with blood as the perfusate and with inhaled concentrations of TCE ranging from 300 ppm to 1270 ppm. One preliminary run was made using Merchant's Solution (Nicolini et al., 1985) as an artificial perfusate and an inhaled TCE concentration of 1750 ppm.

### RESULTS AND DISCUSSION

Using the model of the IVPL to coplot the data points of each run with the model prediction allowed a fit of the data with the model if lung metabolism was set equal to 0.0001 of whole body metabolism for TCE. Figure 4.2-1 shows an example of one run where the inhaled TCE concentration was 1270 ppm and blood was the perfusate. The variable plotted is concentration of TCOH in the perfusate reservoir. A similar plot for the one run conducted with artificial perfusate is shown in Figure 4.2-2. The data for TCA are shown in Figure 4.2-3. Using artificial perfusate, which lacks albumin, avoids the confounding factor of TCA-albumin binding. Runs using blood as the perfusate have produced ambiguous results for TCA because it binds to albumin, and albumin tends to adhere to the IVPL apparatus.

mg Trichloroetnanol/L   (x 10-2)
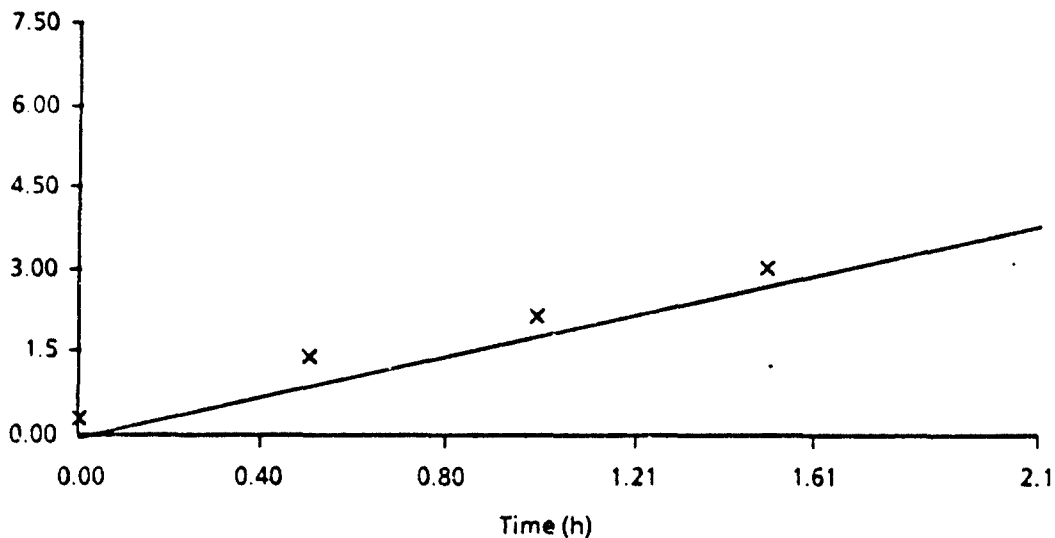


Figure 4.2-1.   Concentration of Trichloroethanol in Blood after Exposure of an IVPL to 1270 ppm of TCE. The continuous line represents the simulation; the individual points represent the actual data.

mg Trichloroethanol/L   (x 10-2)
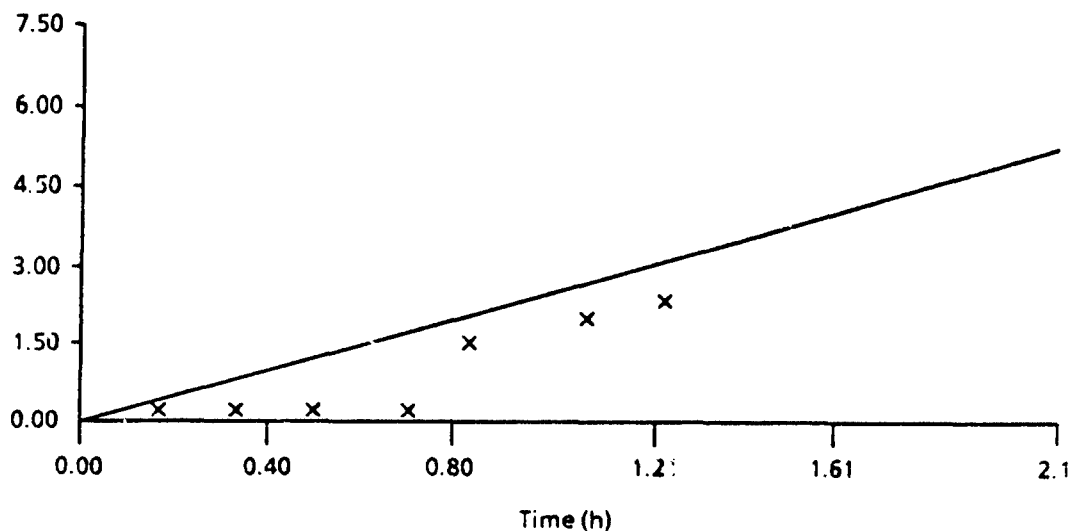


Figure 4.2-2.   Concentration of Trichloroethanol in Artificial Perfusate after Exposure of an IVPL to 1750 ppm of TCE. The continuous line represents the simulation; the individual points represent the actual data.

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mg Trichloroacetic acid/L   ($x$ $10^{-2}$)
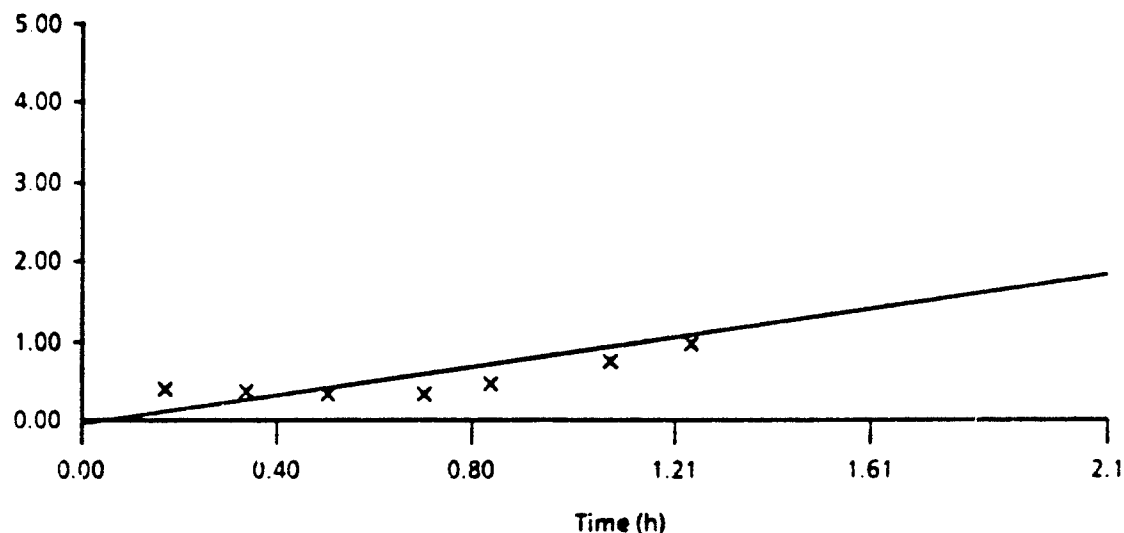


Figure 4.2-3.   Concentration of Trichloroacetic Acid in Artificial Perfusate from the Same Lung Preparation as Shown in Figure 4.2-2. The continuous line represents the simulation; the individual points represent the actual data.

Work is currently planned to measure the metabolism of TCE comparing artificial perfusate to blood. The ratio of TCA to TCOH produced using artificial perfusate will be used to extrapolate the amount of TCA produced when blood is used as the perfusate. The comparison will provide information on whether artificial perfusate can be used instead of blood in future experiments.

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**4.3 DETERMINATION OF THE DERMAL ABSORPTION KINETICS OF SELECTED INSTALLATION RESOTRATION PROGRAM CHEMICALS**

D. Morgan[a] and R. Kutzman

*INTRODUCTION*

Groundwater contamination by a variety of solvents appears to be a growing problem in the civilian arena and at Department of Defense installations. If groundwater is contaminated, it might be presumed that surface water also contains these chemicals. To better understand the potential health hazards from skin contact with contaminated water through hygienic or recreational bathing, studies of the dermal absorption of selected chemicals were conducted. These data will be used to develop and refine models which could then be used to estimate the actual dose subjects would receive when in contact with contaminated water.

This report provides the experimental results from the first three (trichloroethylene, perchloroethylene, and toluene) of 18 chemicals to be studied. Blood concentration of each chemical were measured at selected times during a 24-h period when the chemical was in contact with the skin. Subgroups of animals were treated with either the neat chemical or aqueous solutions of the chemical at saturated, 2/3 saturated, or 1/3 saturated concentrations.

In addition to these kinetic data, skin samples were taken from the treatment area as well as a control area of each animal. The integrity of the skin following exposure to these chemicals and aqueous solutions of them will be assessed by AAMRL/TH personnel.

*METHODS*

Rats were anesthetized and a silastic catheter implanted in the right jugular vein and exteriorized at the back of the neck. Glass exposure cells were attached onto a shaved area on the animal's back with cyanoacrylate adhesive. Velcro wraps were secured around the animals to protect the catheters and the exposure cells. The day after implantation of the jugular catheters, 2 mL of test chemical was added to each exposure cell and the cells were sealed with screw caps containing Teflon® septa.

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Blood samples were withdrawn via the jugular catheters using 1-mL glass syringes. A control blood sample (100 µL) was obtained from each rat just prior to adding the test chemical. Blood samples (100 µL) were withdrawn after exposure for 0.5, 1, 2, 4, 8, 12, and 24 h. Immediately after blood samples were withdrawn, they were placed into labeled 50-mL sampling vials, sealed with crimp tops and Teflon® septa, and placed on ice. Sample vials were stored at 5-10°C until analysis.

Immediately after obtaining the 24-h blood sample, the rats were killed by $CO_2$ asphyxiation. The chemical solution remaining in the exposure cell was withdrawn by syringe and the volume was recorded. The chemical solutions were placed in labeled sample vials, sealed with screw caps containing Teflon® septa, and stored at 5-10°C until chemical analysis. Blood samples were removed from the refrigerator and placed directly into the headspace sampling tray. The sealed sample was heated in the sampler's oil bath to thermodynamic equilibrium. The headspace gas was then flushed onto a 30-m DB-624 capillary gas chromatograph (GC) column. Headspace components were separated and detected by either a flame ionization detector (FID) or an electron capture detector (ECD).

Immediately after removal of the remaining test solutions, the exposure cells were removed, and the skin carefully excised around the exposure area, including a small area of normal unexposed skin. The skin samples were flattened and placed into jars of formalin. After fixation, the samples were removed from the formalin, wrapped in sterile gauze soaked with formalin, and then placed in whirl-pak bags and shipped to WPAFB for electron-microscopic evaluation. The morphological findings will not be presented in this report.

## RESULTS AND DISCUSSION

### Methods Development

The surgical procedures for implanting the jugular catheters were perfected prior to beginning the dermal exposures. Very few animals have been lost because of surgical complications. The major problem encountered has been blockage of the catheters caused by blood clots. Once blockage has occurred, it is very difficult to clear the catheter, and subsequent blood samples can not be obtained from the animal. This problem was partially overcome by filling the catheters after each blood sample with fresh saline containing 30 units heparin/mL. This procedure prevented clotting within the catheter; however, some clots still occurred inside the vein at the tip of the catheter. To slow the clotting time of the blood (and to maintain the blood volume) 100 µL of heparin/saline solution was injected back into the animal after implanting the catheter and after obtaining each 100-µL blood sample. These preventive measures have helped to maintain the patency of the jugular catheters. Of the initial 10 catheterized rats per group, usually seven to eight catheters are still patent after the 24-h sampling time.

A number of blood samples were lost initially due to storage at -70°C. This storage temperature was selected to lower the vapor pressure of the test chemical and to ensure that the chemical remained dissolved in the blood samples. However, after storage at -70°C for only 24 h, no test chemical could be detected in the sample vials. It appeared that the extreme cold caused the crimp top and septa to contract, and after thawing, the vials were no longer airtight. When the vials were heated in the headspace analyzer, the chemical in the headspace leaked from the vial and was lost. This unexpected problem was easily resolved by storing samples at 5-10°C instead of -70°C. Samples have been found to remain stable at 5-10°C for at least three weeks.

Because of the reported ability of halogenated compounds to adsorb or penetrate Teflon®, an experiment was performed to determine if the chemical would be lost from sample vials that were sealed with Teflon® septa. Samples of an aqueous trichloroethylene (TCE) stock solution of known concentration were dispensed into 10 sample vials and sealed with crimp tops containing Teflon® septa. Five samples were stored at room temperature (23°C) and five at 5°C, and the TCE concentrations were measured after storage for 1 and 21 days. As a control, a fresh sample of stock TCE solution was also analyzed.

As shown in Table 4.3-1, the fresh stock solution differed by 4.1% from the value measured the day before. Samples stored for one day at 23°C or 5°C varied from initial measurements by 1.1% and 1.5%, respectively. After 21 days, samples stored at 23°C and 5°C differed by 6.1% and 3.5% from initial measurements. Because the daily drifts in the GC system result in 4.1% variations, these differences detected in samples stored at 5°C are not considered significant.

TABLE 4.3-1.  DETECTION OF TRICHLOROETHYLENE FOLLOWING STORAGE OF
SAMPLES AT 5°AND 23°C

| Storage Time | % Difference from Initial Measurements | | |
|---|---|---|---|
| | Fresh Stock | 23°C | 5°C |
| 1 Day | 4.1 | 1.1 | 1.5 |
| 21 Days | – | 6.1 | 3.5 |

The levels of Installation Restoration Program (IRP) chemicals present in blood after various durations of dermal exposure were determined by headspace GC using ECD or FID. Initial methods development was necessary to establish analytical conditions (e.g., headspace equilibrium temperatures, column temperatures, run times) for each chemical and to determine the range of chemical concentrations expected after exposure to neat compound as well as aqueous solutions. For aqueous solutions of chemicals analyzed by FID, cryofocusing was employed, which permits multiple injections to be made from a single sample vial while the oven is at -80°C. No chromatography occurs until the oven is heated following the third injection from the sample vial.

Cryofocusing enhanced the signal and permitted detection at or around 15 ppb (ng/mL blood). IRP chemicals with multiple halogen groups were analyzed by ECD and ramping the oven temperatures from about 40°C to 150°C. Run times were typically about 12 min for FID and 8 min for ECD.

Immediately after the 24-h exposure, the chemical remaining in the exposure cell was removed, the volume recorded, and the solution placed in one-dram glass vials sealed with screw caps containing Teflon® septa. Samples were stored at 5-10°C until analysis. Data were not collected for exposure cells that were observed to be leaking. Exposure cells that lost considerably more chemical solution than other cells in that group were assumed to have leaked and were not included.

The volumes of chemical remaining in the exposure cells after the 24-h exposure were dependent upon the concentration of the exposure solution. The volumes of neat solutions decreased most, followed by the saturated, 2/3-saturated, and 1/3-saturated aqueous solutions, respectively. None of the sample volumes were observed to increase, suggesting that body fluids (e.g., serum) were probably not exuded through the skin into the exposure cell.

Chemical analyses of the aqueous solutions remaining in the exposure cells after 24 h of exposure revealed that less than 1% of the chemical was still present (<1 ppm). These results suggested that either 99% of the chemical was absorbed from the solution by the animal, was lost from the sample vials before analysis, or escaped from the exposure cell during the exposure.

The chemical solution remaining was transferred to 4-mL sample vials and stored for analysis. Because the exposure vials were not completely filled, the chemicals could have come out of the aqueous solutions and into the vial headspace. The chemical would then have been lost when the vial was opened. To prevent the dissolved aqueous chemicals from moving out of solution and into the headspace, the sample vials were prefilled with about 2 mL methanol, which acted to stabilize the dissolved chemical. However, the levels of chemical remaining in samples prepared in this manner were still below 1 ppm, suggesting that the chemicals were either absorbed by the animals or lost from the exposure cells during the exposure.

Because the exposure cells were only partially filled with the chemical solution (2 mL), leaving some headspace, the chemical could have come out of solution and been trapped in this headspace. Movement of the chemical from the solution to the headspace could possibly be facilitated by the increased temperature contributed by the animal's body. When the exposure cell was opened to remove the solution, the headspace containing the chemical would be lost. The decrease in the amount of dissolved chemical would also explain the decreases in blood levels of chemical with increased time of exposure to aqueous solutions.

To determine if the chemical was being lost in this manner, three rats were exposed to 2 mL of 2/3 saturated toluene by the standard procedure (2 mL/exposure cell), and three rats were exposed

by filling the exposure cells completely with 2/3 saturated toluene (~3.4 mL/exposure cell), thereby eliminating the headspace. Solutions were removed after 24 h, the volumes recorded, and the samples transferred to vials containing 2 mL of methanol. As a control, three 2-mL samples of the stock 2/3 saturated toluene were added to vials containing methanol, and all samples were analyzed. Toluene levels in all six samples from exposed animals were less than 1 ppm. Completely filling the exposure cells did not prevent loss of the chemical from the solution, suggesting that the chemical was not lost in the exposure cell headspace. Control samples contained about 220 ppm toluene, demonstrating that the chemical was not being lost from the sample vial and analytical methods were not the problem. These preliminary experiments suggested that the chemical was being absorbed by the animal.

## Blood Levels of Trichloroethylene

During dermal exposure to neat TCE, blood TCE levels attained peak concentrations after 0.5 h of exposure (Figure 4.3-1). Blood levels remained elevated and did not change during the remainder of the 24-h exposure period.

Dermal exposure to aqueous solutions of TCE also resulted in rapid increases in blood TCE levels which peaked after 1 h of exposure to saturated TCE, after 0.5 h to 2/3-saturated and after 1 h to 1/3-saturated TCE (Figure 4.3-2). Blood levels decreased to control levels by 8 h for saturated TCE, and by 12 h for the 1/3- and 2/3-saturated aqueous solutions.

Control blood samples had relatively high TCE levels. However, it was determined that these high levels were caused by slight sample carryover from TCE standards that were run just prior to the control blood samples. Actual blood levels of TCE in control rats were not as high as indicated by these data.

## Blood Levels of Perchloroethylene

Perchloroethylene (PCE) exposure resulted in peak blood levels after exposure to neat chemical for 0.5 hour (Figure 4.3-3). Blood levels appeared to decrease slightly with exposure time.

Exposure to aqueous PCE solutions resulted in blood levels that varied considerably (Figure 4.3-4). Peak blood PCE levels were observed after exposure for 0.5 h to saturated PCE, 1 h to 2/3-saturated PCE, and after 12 h to 1/3-saturated PCE. During exposure to saturated PCE, blood levels appeared to follow a biphasic curve with peaks occurring after 0.5 and 8 h of exposure. This trend was not observed during exposure to the 2/3- and 1/3-saturated PCE. However, exposure to 2/3-saturated PCE produced a higher peak blood level than exposure to saturated PCE.

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Figure 4.3-1.  Concentrations of Trichloroethylene in the Blood of Rats Exposed to the Neat Chemical for 24 h.  Each point and bar represents the mean ( ± s.e.) of at least seven animals.



Figure 4.3-2.  Concentrations of Trichloroethylene in the Blood of Rats Exposed to Aqueous Solutions at Saturated, 2/3-Saturated, or 1/3-Saturated Concentration for 24 h. Each point and bar represents the mean ( ± s.e.) of at least seven animals.
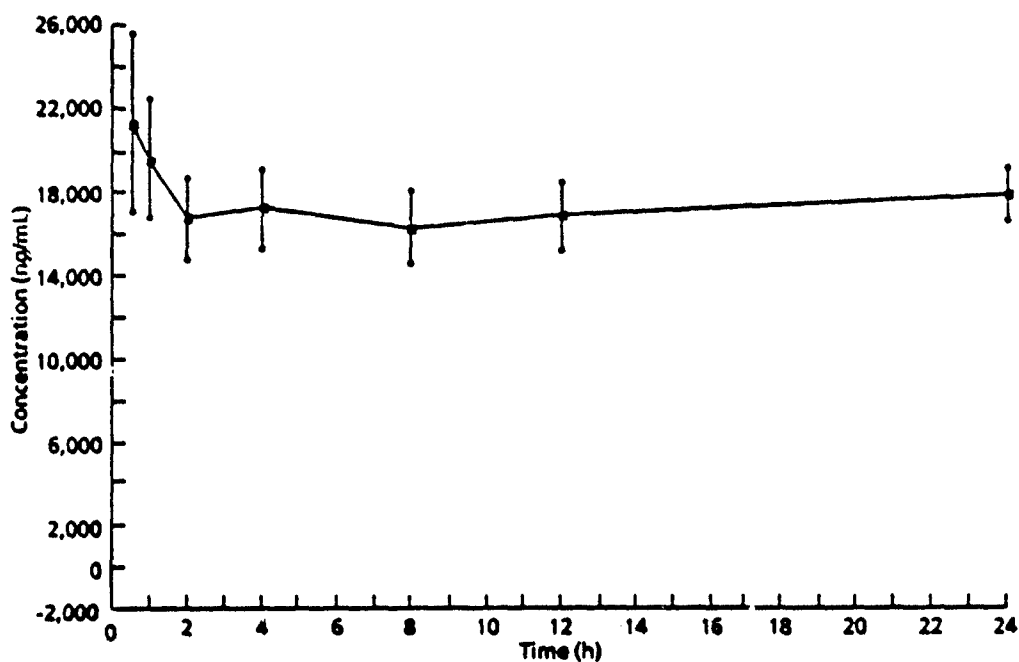
Figure 4.3-3.  Concentrations of Perchloroethylene in the Blood of Rats Exposed to the Neat Chemical for 24 h.  Each point and bar represents the mean ( ± s.e.) of at least eight animals.
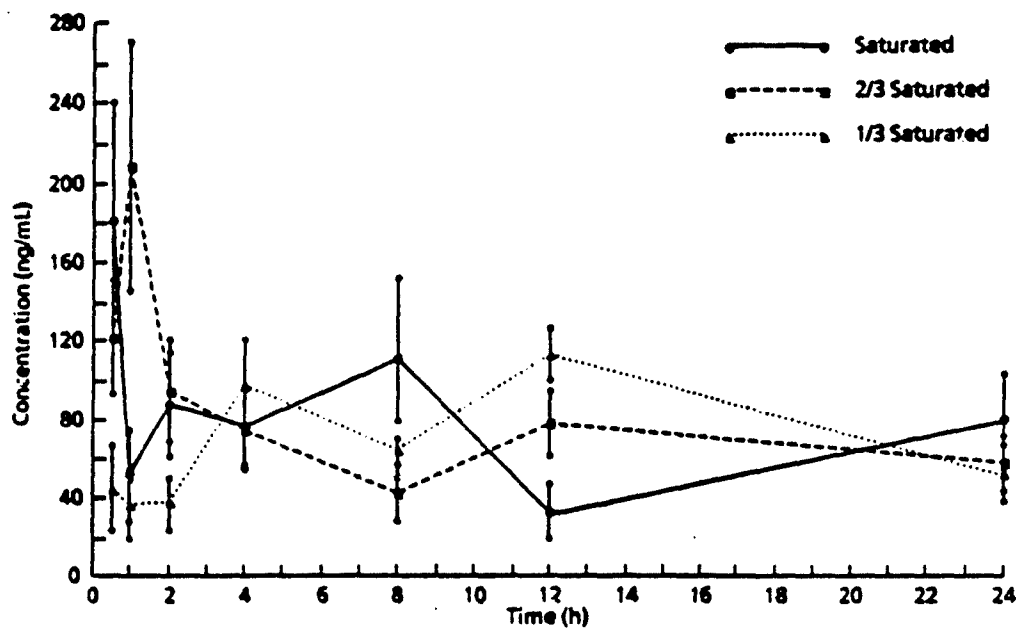


Figure 4.3-4.  Concentrations of Perchloroethylene in the Blood of Rats Exposed to Aqueous Solutions at Saturated, 2/3-Saturated, or 1/3-Saturated Concentration for 24 h.  Each point and bar represents the mean ( ± s.e.) of at least eignt animals.

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## Blood Levels of Toluene

Exposure to neat toluene resulted in a peak blood toluene level after exposure for 1 h (Figure 4.3-5). Blood toluene levels decreased with time and appeared to remain unchanged during exposure for 4 to 24 h.

Peak blood levels were observed after exposure for 12 h to saturated toluene, 2 h to 2/3-saturated, and 2 h to 1/3-saturated toluene (Figure 4.3-6). Exposure to 1/3-saturated toluene produced higher peak blood levels than exposure to 2/3-saturated toluene. The highest blood levels of toluene resulted from exposure to saturated toluene. Blood levels of toluene after exposure to aqueous solutions were very low and near the detection limit. Measurement of these very low levels may have contributed to some of the variation in these data.

## CONCLUSIONS

The objectives of this study were completed for the first three of 18 IRP chemicals to be studied. Methods were established for performing dermal exposures, blood sampling, and gas chromatographic analyses of IRP chemicals in blood. Blood levels of TCE, PCE, and toluene were reported for dermal exposures to neat, saturated, 2/3-saturated, and 1/3-saturated aqueous solutions for 0, 0.5, 1, 2, 4, 8, 12, and 24 h. Although blood levels after aqueous chemical exposures were all quite low, reliable values were obtained as low as 50 ppb in blood.

After exposure for 24 h, the volume and concentrations of the chemical solutions remaining in the exposure cells were measured. In general, the volumes of the remaining solutions were inversely related to the initial concentrations of the solutions. The volumes of neat solutions decreased the most, followed by the saturated, 2/3-saturated, and 1/3-saturated aqueous solutions. The concentrations of these solutions were all less than 1 ppm. Based upon preliminary experiments, it appeared that the chemicals were depleted from the solutions due to absorption by the tissues of the animals.
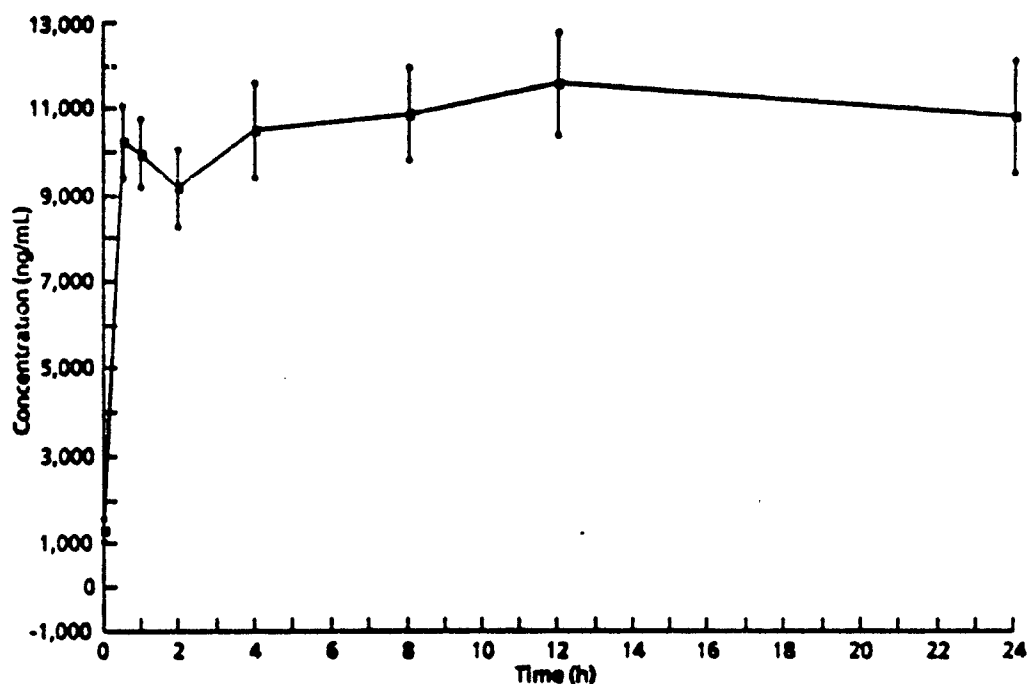
32
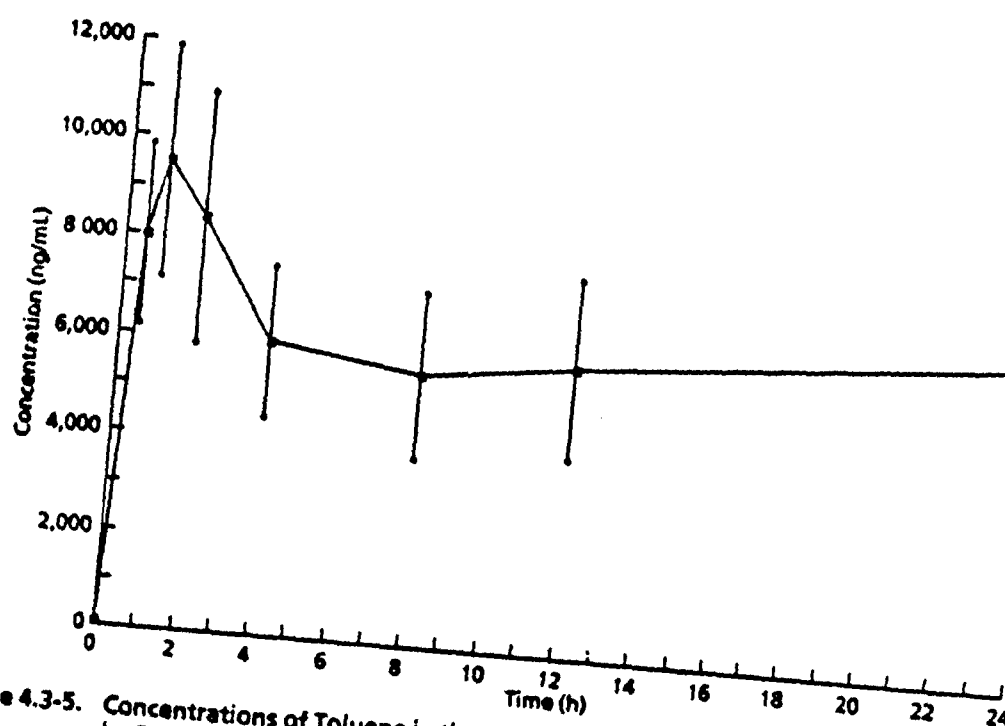
Figure 4.3-5. Concentrations of Toluene in the Blood of Rats Exposed to the Neat Chemical for 24 h. Each point and bar represents the mean (± s.e.) of at least eight animals.
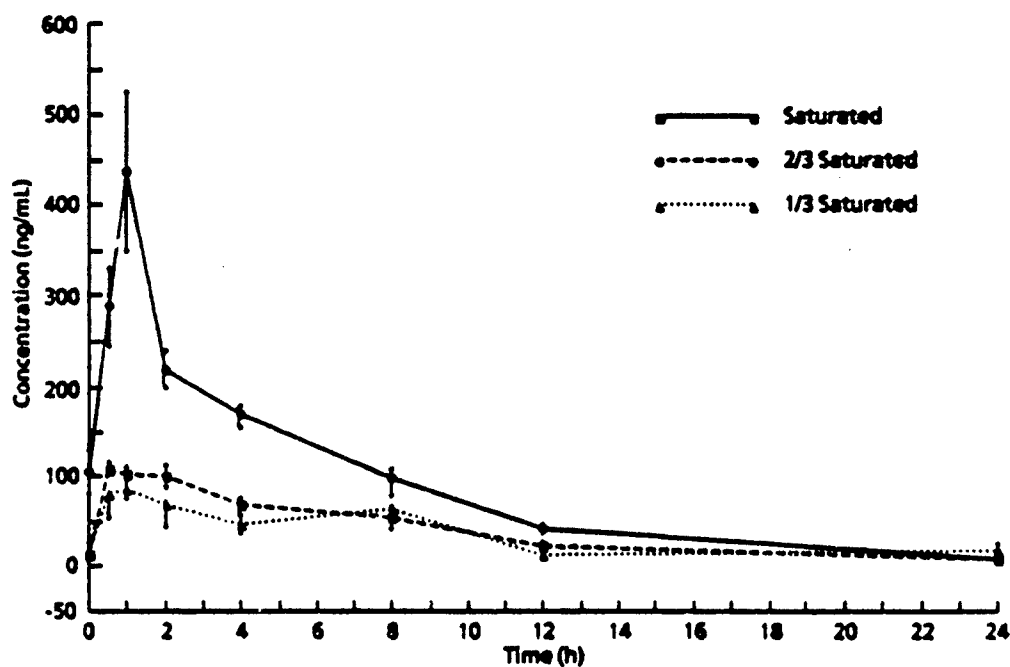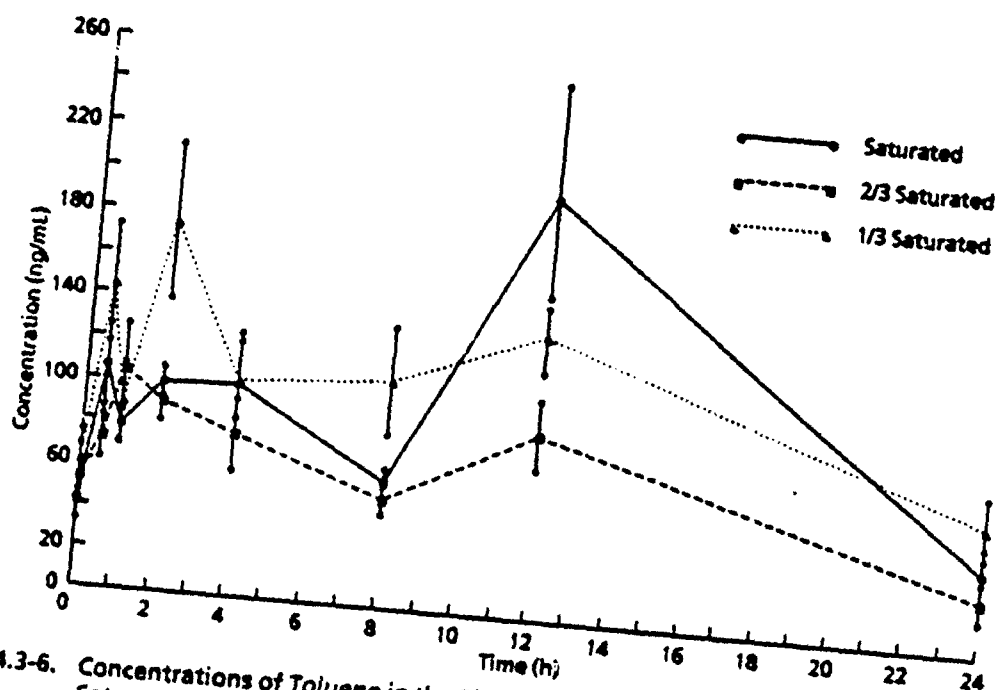


Figure 4.3-6. Concentrations of Toluene in the Blood of Rats Exposed to Aqueous Solutions at Saturated, 2/3-Saturated, or 1/3-Saturated Concentration for 24 h. Each point and bar represents the mean (± s.e.) of at least eight animals.

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## 4.4 THE ACUTE AND SUBCHRONIC TOXICITY TESTING OF INSTALLATION RESTORATION PROGRAM SAMPLES

E. Kinkead, S. Henry, B. Culpepper, and R. Kutzman

### INTRODUCTION

The Air Force is conducting a large-scale program to identify, characterize, and manage hazardous waste sites at Air Force installations worldwide. Although long-term monitoring, risk assessment, and abatement efforts are included and well orchestrated within the overall management program, many installations have short-term, site-specific problems requiring immediate abatement action (e.g., open discharges of landfill leachate to areas with public access).

This study involves acute and subchronic toxicity testing on a series of leachate samples taken at Wright-Patterson Air Force Base. The samples were collected at multiple discharge points under various conditions. The Air Force has split the samples, sending a portion to another facility for chemical analysis. Acute testing includes oral and dermal toxicity, eye and skin irritation, and skin sensitization. Subchronic testing consists of 14-day repeated oral and 14-day repeated dermal dosing. The study protocol follows EPA guidelines. Existing alternative methods to animal testing are inadequate for this study.

Results of these toxicity studies combined with chemical analyses of the leachate are intended to provide a basis for the evaluation of potential health hazards posed by the effluents.

### MATERIALS

#### Test Agents

Three Installation Restoration Program (IRP) samples have been provided to date by the Air Force. The identified samples (Table 4.4.1) received at the Toxic Hazards Research Unit were refrigerated at 2 to 4°C and continuously stirred, using a magnetic stir plate/bar apparatus, throughout the testing period. Archive samples of each test material were collected and placed in storage at ambient temperature.

**TABLE 4.4-1. TEST SAMPLE IDENTIFICATION**

| Sample Collection Date | Sample Identification |
|---|---|
| 5 February 1987 | L-3, 1-5-87 |
| 24 April 1987 | Landfill #8 |
| 24 April 1987 | Landfill #10 |

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## Animals

New Zealand White rabbits (Clerco Research Farms, Cincinnati, OH) weighing between 2 and 3 kg were used in the dermal toxicity, eye irritation, and skin irritation studies. Male and female Fischer-344 rats (Charles River Breeding Lab, Kingston, NY) weighing 150 to 250 g and 125 to 250 g, respectively, were used to evaluate oral toxicity of the test compounds. Male, albino, Hartley strain guinea pigs (Murphy Breeding Laboratory, Inc., Plainfield, IN), weighing between 200 and 250 g upon receipt, were used in the sensitization tests.

Quality control assessment during a two-week quarantine period confirmed the acceptable health of the proposed study animals. The rabbits and guinea pigs were housed individually, the guinea pigs in plastic cages with wood chip bedding, and the rabbits in wire-bottom stainless-steel cages. The rats were group housed, two to three per plastic cage with wood chip bedding. Water and feed (Purina Rabbit Chow #5320; Purina Formulab #5015 for guinea pigs and Purina Formulab #5008 for rats) were available *ad libitum*, except that rats in acute and subchronic oral toxicity studies were fasted for 16 h prior to each dose. All animals were on a 12-h interval light/dark cycle.

## Evaluation Scales

Evaluations of skin and ocular lesions were based on the scoring system of Draize (1959). The scales for scoring skin and eye reactions are given in Appendices 1 and 2, respectively. The sensitization testing procedure is a modification of the Maguire (1973) method as described by Horton et al. (1981). Appendix 3 contains the method used to score erythematous and edematous reactions following dosing. Test materials are then classified as to sensitization potential using Appendix 4.

## EXPERIMENTAL APPROACH

### Acute Oral Toxicity

Ten male and ten female Fischer-344 rats were fasted for 16 h before dosing. Five rats of each sex were dosed with the test material while an equal number of control animals were dosed with laboratory-pure water and maintained for body weight and pathology comparison. The 5.0 g/kg-limit test dose was administered by oral gavage. Just prior to dosing, each rat was weighed to determine dose volume.

Surviving rats were weighed at 1, 2, 3, 7, 10, and 14 days. Rats were examined daily, and any signs of toxicity were recorded. All animals that were alive at the end of the observation period were sacrificed for gross pathological examination.

*Eye Irritation*

For each sample to be tested, the eyes of nine albino rabbits were stained with fluorescein and examined to ensure absence of lesions or injury. A topical anesthetic (OPTHETIC, Proparacaine HCl 0.5%; Allergan Pharmaceuticals, Inc., Irvine, CA) was instilled in both eyes of each rabbit approximately 2 min before applying the test material. One-tenth of a milliliter of test material was then applied to one eye of each rabbit; the other eye was left untreated to serve as a control. In three rabbits, the treated eye was flushed for 1 min with lukewarm water, starting 30 s after instilling the test material; while in six rabbits, the treated eye was not washed. Eyes were examined for gross signs of irritation at 1, 24, 48, and 72 h following application. Scoring of irritative effects was based on the Draize (1959) method in which the total score for the eye is the sum of all scores obtained for the cornea, iris, and conjunctiva. No gross pathology or histopathology was performed.

*Skin Irritation*

A patch test was used to determine the degree of primary skin irritation on intact skin of albino rabbits. All hair was clipped from the backs and sides of six rabbits 24 h prior to exposure to allow the skin to recover from any abrasion resulting from the clipping. One-half milliliter of undiluted test material was applied to an ~3 cm²-area on the back of each rabbit. This area was covered by a 3 cm²-surgical gauze, two single layers thick. The patch was held in place with surgical adhesive tape, and the entire shaved area was covered with dental dam and secured with an elastic bandage and adhesive tape. After 4 h, the wrap and patches were removed, excess material was wiped from the skin, and each test area was evaluated for irritation using the Draize (1959) table as a reference. Irritation evaluations were also performed at 24, 48, and 72 h. No gross pathology or histopathology was performed.

*Sensitization*

Prior to the actual sensitization testing, a separate group of albino guinea pigs was used to confirm that the neat test samples were not irritating to guinea pig skin. In addition, 0.1 mL of undiluted test material was applied to the clipped left flank of the study animals to identify hypersensitive individuals and remove them from the study.

For each test sample, the upper backs of ten guinea pigs were clipped and chemically depilated (Surgex® Hair Remover Cream, Sparta Instrument Corp., Hayward, CA) prior to the first application. The shoulder areas of the study animals were then wrapped with a latex bandage to outline the treatment area and to provide a base for holding cover bandages in place. One-tenth milliliter of test material was then topically applied to a 1.5-cm²-area and covered by gauze, dental dam, and adhesive tape. This procedure was repeated on Mondays, Wednesdays, and Fridays until a total of four sensitizing treatments was applied. Along with the third sensitizing treatment, 0.2 mL of a 50% aqueous dilution of Freund's (Bacto Adjuvant Complete, Freund; Difco Laboratories,

Detroit. MI) adjuvant per animal was injected intradermally using two or three sites next to the test area. Following the fourth application, the animals were rested for two weeks.

Following two weeks of rest, the left flank was clipped and challenged with 0.1 mL of test material. The challenge applications were not occluded. The animals were then examined for signs of a sensitization reaction at 4, 12, 24, and 48 h. Any animal eliciting a score of two or more at the challenge site at the 48-h scoring was rated a positive responder. In scoring this test, it should be noted that the important statistic is the frequency of the reaction (Appendix 4).

### Subchronic Oral Toxicity (14-Day)

Ten male and ten female Fischer-344 rats were used to test each material. These rats were dosed with 5.0 g test agent/kg (the same dose used in acute oral toxicity testing), while an equal number of male and female rats were dosed with 5.0 g laboratory-pure water/kg. The animals were dosed daily for 14 consecutive days, including weekends. The dosage volumes were calculated from individual body weights and adjusted weekly. Administration by gavage was performed at the same time each day and followed a 16-h fast. Food was provided immediately after dosing and water was available ad libitum. Test and control groups were sacrificed on the day following the 14th dose.

Body weights were measured on days 0 (immediately prior to the first dose), 7, and 14. Blood samples (limited to five rats of each sex per test or control group) were collected from the posterior vena cava at sacrifice for the assays listed in Table 4.4-2.

TABLE 4.4-2. WHOLE BLOOD AND SERUM CHEMISTRY ASSAYS FOR
SUBCHRONIC ORAL TOXICITY STUDY

| Whole Blood Assays | Serum Chemistry Assays |
| --- | --- |
| White blood cell count | Chloride |
| Red blood cell count | Blood urea nitrogen |
| Hemoglobin | Creatinine |
| Mean corpuscular volume | Alkaline phosphatase |
| Hematocrit | Calcium |
| Mean corpuscular hemoglobin | Sodium |
| Differential leucocyte count | Potassium |
| Mean corpuscular hemoglobin concentration | Serum glutamic-oxalacetic transaminase |
| Morphology of blood smears | Total protein |
| | Total bilirubin |
| | Serum glutamic-pyruvic transaminase |

At sacrifice, the following organ weights were recorded: adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, testes, and thymus. A gross pathologic examination was performed on each rat, and selected tissues were taken for histopathologic examination (Table 4.4-3).

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**TABLE 4.4-3. TISSUES COLLECTED FOR HISTOPATHOLOGIC
EXAMINATION FROM SUBCHRONIC ORAL TOXICITY STUDY**

| | | |
|---|---|---|
| Gross lesions | Urinary bladder | Uterus |
| Kidneys | Mandibular lymph nodes | Esophagus |
| Trachea | Mesenteric lymph nodes | Stomach |
| Heart | Sciatic nerve | Cecum |
| Liver | Brain | Colon |
| Spleen | Lungs | Rectum |
| Duodenum | Adrenals | Sternum |
| Jejunum | Pancreas | Thymus |
| Ileum | Gonads | Skeletal muscle |

### Subchronic Dermal Toxicity (14-Day)

Ten male and ten female New Zealand White rabbits were used to test each material. These rabbits were dosed with 2.0 g test agent/kg, while an equal number of male and female rabbits were dosed with 2.0 g laboratory-pure water/kg. The treated area was covered by 8-ply gauze squares, which in turn were covered by a clear plastic wrap surrounding the entire midsection of the body and, finally, by a layer of elastic tape. The test material was held in contact with the skin for 6 h per day, after which the tape, plastic wrap, and gauze were removed, and residual material was wiped from the skin. The animals were dosed daily for 14 consecutive days, including weekends. The dosage volumes were calculated from individual body weights and adjusted weekly. Food and water were available ad libitum. Test and control groups were sacrificed on the day following the 14th dose.

Body weights were measured on days 0 (immediately prior to the first dose), 7, and 14. Blood samples (limited to five rabbits of each sex per test or control group) were collected twice, one day prior to the first dosing and at sacrifice, from the marginal ear vein. Assays performed on rabbit blood samples are listed in Table 4.4-4.

**TABLE 4.4-4. WHOLE BLOOD AND SERUM CHEMISTRY ASSAYS FOR
SUBCHRONIC DERMAL TOXICITY STUDY**

| Whole Blood Assays | Serum Chemistry Assays |
|---|---|
| Hematocrit | Creatinine |
| Hemoglobin | Chloride |
| Red blood cell count | Blood urea nitrogen |
| Total & differential leucocyte count | Total protein |
| | Alkaline phosphatase |
| | Serum glutamic-pyruvic transaminase |
| | Serum glutamic-oxalacetic transaminase |
| | Lactate dehydrogenase |

At sacrifice, the following organ weights were recorded: adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, testes, and thymus. A gross pathologic examination was performed on each rabbit, and selected tissues were taken for histopathologic examination (Table 4.4-5).

### TABLE 4.4 5. TISSUES COLLECTED FOR HISTOPATHOLOGIC EXAMINATION FROM SUBCHRONIC DERMAL TOXICITY STUDY

| Gross lesions | Normal and treated skin | Uterus |
|---|---|---|
| Lungs | Mandibular lymph nodes | Esophagus |
| Trachea | Mesenteric lymph nodes | Stomach |
| Heart | Gall bladder | Cecum |
| Liver | Thymus | Colon |
| Spleen | Brain | Rectum |
| Duodenum | Kidneys | Sternum |
| Jejunum | Adrenals | Sciatic nerve |
| Ileum | Pancreas | Skeletal muscle |
| Urinary bladder | Gonads | |

## Statistics

A repeated measures test is being used to compare body weights of acute oral toxicity test animals against controls (Dixon, 1985; Barcikowski, 1983). Subchronic oral and dermal toxicity data are being statistically analyzed as follows.

| | |
|---|---|
| Body Weight | repeated multivariate analysis of variance with Scheffe pairwise comparisons (Dixon, 1985; Barcikowski, 1983). |
| Hematology | a two-factorial analysis of variance with multiple comparisons (Dixon, 1985; Barcikowski, 1983). |
| Clinical Chemistry | a two-factorial analysis of variance with multiple comparisons (Dixon, 1985; Barcikowski, 1983). |
| Organ Weights | a two-factorial analysis of variance with multiple comparisons (Dixon, 1985; Barcikowski, 1983). |
| Histopathology | one of the following nonparametric tests: Fischer's Exact test or, if determined not valid, Yates' Corrected Chi-square (Zar, 1974). |

## RESULTS

### Acute Oral Toxicity

A total of 30 F-344 rats (five male and five female per test material) were orally dosed with the IRP leachate samples. Twenty control rats (five male and five female per control group) were dosed concurrently with water. Since the Landfill #8 (L-8) and Landfill #10 (L-10) samples were received on the same day, they were tested simultaneously using a single control group.

During the 14-day observation period, all animals gained weight (Figures 4.4-1 and 4.4-2) and showed no clinical signs of toxicity. Body weights of the control animals and the treatment animals, on day 0 (first day of study) and on day 14 (final day of study), did not differ significantly ($p < 0.05$). Gross pathological examination of all animals at the conclusion of the study did not show dose-related lesions.
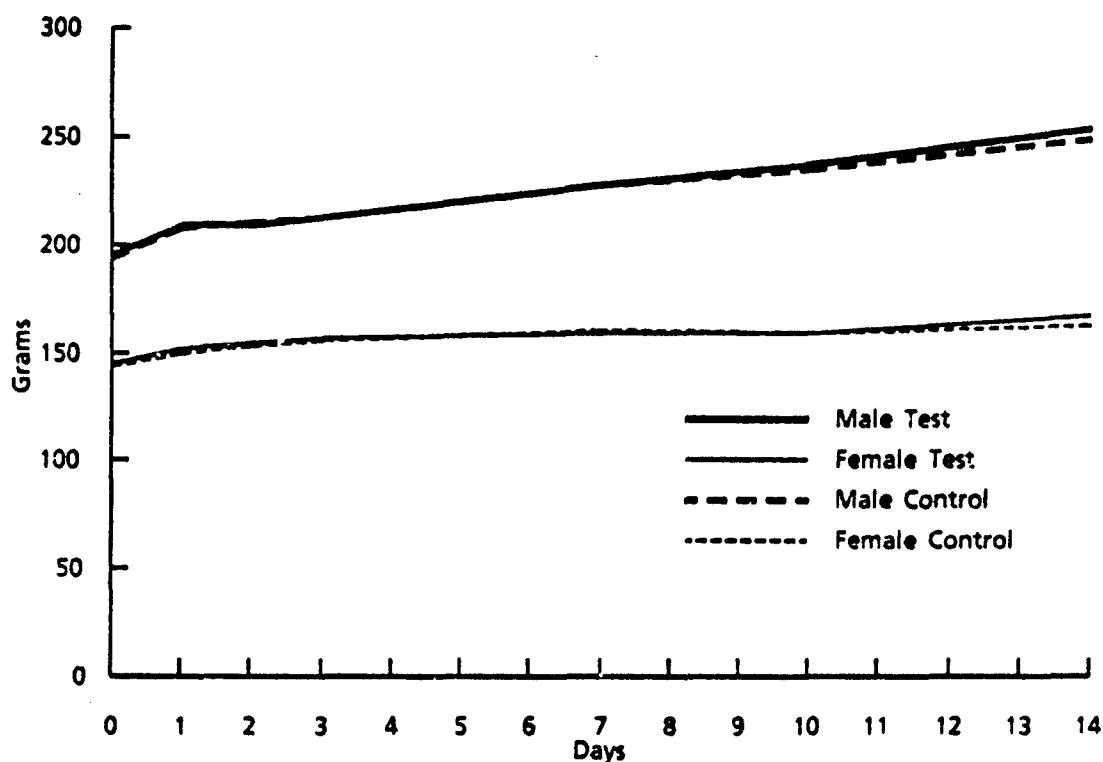
**Figure 4.4-1.** Body Weights for Test and Control Male and Female F-344 Rats Orally Dosed with Test Material L-3.

### Eye Irritation

Prior to initiation of skin and eye irritation studies, the pH of the test agents was determined with the intention that predictably corrosive materials (pH of less than 2 or greater than 11.5) would not be tested. The pH of each material was neutral, within the range of 6.0 to 7.0.

Twenty-seven rabbits were used (nine per test sample) in this portion of the study. Mild conjunctival redness, as well as mild corneal abrasions were observed in the eyes of rabbits treated with the L-3 sample. Similar, but of lesser intensity, conjunctival and corneal effects were present in the rabbit eyes treated with the L-8 and L-10 samples. None of the rabbits showed any inflammation or irritation of the iris. Washing the eyes did not reduce the mildly irritative effects of the samples.

### Skin Irritation

No erythema, edema, or necrosis was observed in any of the rabbits upon examination immediately following 4-h dermal contact with the test agents. Subsequent observations at 24, 48, and 72 h also were negative in all respects.
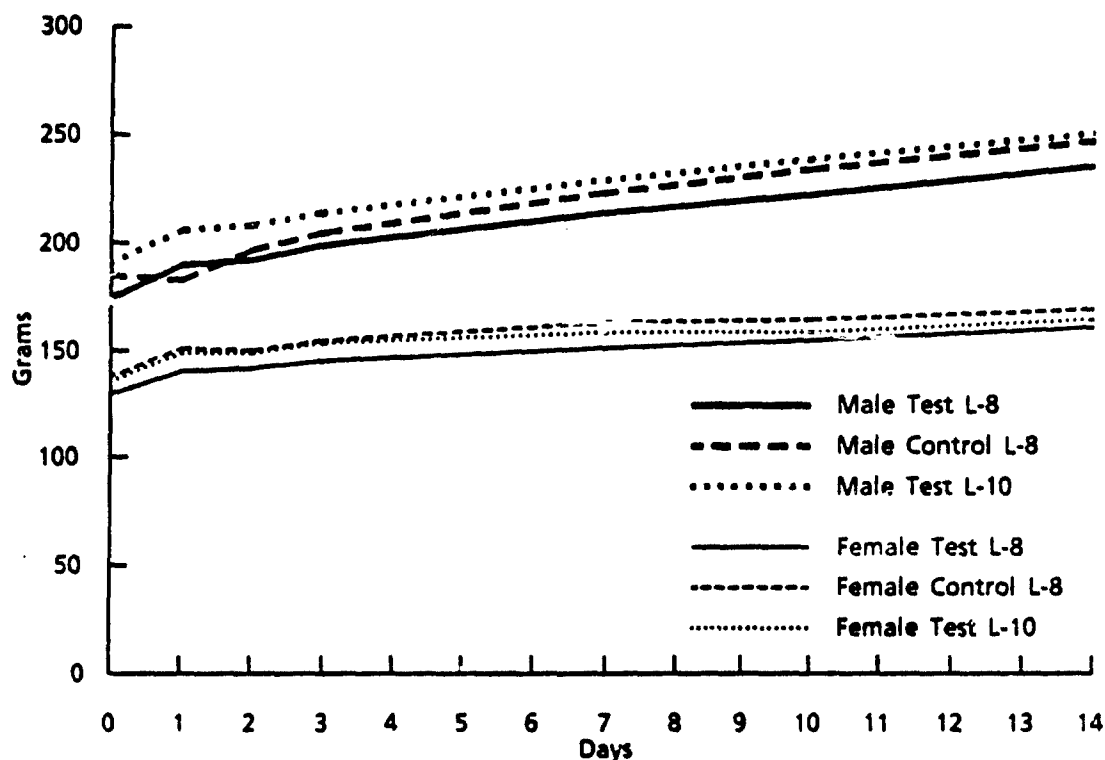
**Figure 4.4-2.** Body Weights for Test and Control Male and Female F-344 Rats Orally Dosed with Test Materials L-8 and L-10.

## Sensitization

Thirty guinea pigs (ten per test material) were challenged with the IRP leachates two weeks after the sensitization treatment. None of the animals displayed any signs of sensitivity to the test agents (i.e., no erythema or edema was present at the challenge sites at 4, 12, 24, or 48 h).

## Subchronic Oral Toxicity

A total of 100 F-344 rats were orally dosed for two weeks. Ten males and ten females were used for each of three test and two control groups. One control group was used for studies on sample L-3, while the second control group was used for studies on both samples L-8 and L-10, which were received and tested concurrently. No toxic signs were noted in any of the rats during the 14-day dosing period. The mean body weights of each test and control group are listed in Table 4.4-6.

Organ weights were recorded at necropsy (Tables 4.4-7 and 4.4-8). No statistically significant differences were noted in the organ weights or the organ/body weight ratios in any of the test groups when compared to their respective control group. Gross pathological examination of the animals at necropsy failed to reveal any dose-related lesions.

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**TABLE 4.4-6. MEAN BODY WEIGHTS (g) OF F-344 RATS IN THE SUBCHRONIC ORAL TOXICITY STUDY**

| Test/Control Group (n = 10) | Male Rats | | | | Female Rats | | |
|---|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 14 | | Day 0 | Day 7 | Day 14 |
| Control | 199 | 196 | 208 | | 142 | 143 | 147 |
| (± S.E.M.) | (± 2.8) | (± 3.0) | (± 4.1) | | (± 1.1) | (± 0.7) | (± 1.1) |
| L-3 | 198 | 195 | 202 | | 142 | 143 | 146 |
| (± S.E.M.) | (± 1.9) | (± 1.9) | (± 2.0) | | (± 1.8) | (± 2.2) | (± 2.6) |
| Control | 196 | 193 | 199 | | 139 | 137 | 142 |
| (± S.E.M.) | (± 3.0) | (± 3.3) | (± 3.8) | | (± 1.1) | (± 1.3) | (± 1.6) |
| L-8 | 193 | 192 | 193 | | 142 | 138 | 139 |
| (± S.E.M.) | (± 2.2) | (± 2.1) | (± 3.8) | | (± 1.5) | (± 1.0) | (± 0.9) |
| L-10 | 197 | 191 | 197 | | 143 | 135 | 139 |
| (± S.E.M.) | (± 2.6) | (± 2.7) | (± 3.9) | | (± 1.7) | (± 2.0) | (± 1.6) |

**TABLE 4.4-7. MEAN ORGAN WEIGHTS (g) AND ORGAN TO BODY WEIGHT RATIOS (g/100 g Body Wt) OF MALE RATS FOLLOWING 14 DAYS OF ORAL ADMINISTRATION OF 5.0 g/kg IRP LEACHATE SAMPLES**

| Organ | Sample L-3 | | Sample L-8 and L-10 | | |
|---|---|---|---|---|---|
| | Test | Control | L-8 Test | L-10 Test | Control |
| | n = 10 | n = 10 | n = 9 | n = 10 | n = 10 |
| Kidneys | 1.615 | 1.659 | 1.591 | 1.579 | 1.571 |
| (Ratio) | (0.80) | (0.80) | (0.81) | (0.80) | (0.79) |
| Heart | 0.706 | 0.709 | 0.660 | 0.654 | 0.699 |
| (Ratio) | (0.35) | (0.34) | (0.34) | (0.33) | (0.35) |
| Testes | 2.863 | 2.873 | 2.570 | 2.584 | 2.528 |
| (Ratio) | (1.42) | (1.38) | (1.31) | (1.31) | (1.27) |
| Brain | 1.802 | 1.806 | 1.756 | 1.747 | 1.729 |
| (Ratio) | (0.89) | (0.87) | (0.90) | (0.89) | (0.87) |
| Liver | 7.488 | 7.957 | 6.192 | 6.430 | 6.556 |
| (Ratio) | (3.70) | (3.83) | (3.16) | (3.26) | (3.29) |
| Spleen | 0.476 | 0.477 | 0.481 | 0.477 | 0.481 |
| (Ratio) | (0.24) | (0.23) | (0.25) | (0.24) | (0.24) |
| Thymus | 0.271 | 0.256 | 0.285 | 0.262 | 0.269 |
| (Ratio) | (0.13) | (0.12) | (0.15) | (0.13) | (0.14) |
| Lungs | 1.441 | 1.505 | 1.629 | 1.814 | 1.859 |
| (Ratio) | (0.71) | (0.73) | (0.63) | (0.92) | (0.93) |
| Adrenals | 0.074 | 0.078 | 0.062 | 0.063 | 0.064 |
| (Ratio) | (0.04) | (0.04) | (0.03) | (0.03) | (0.03) |
| Body Weight (g) | 202.3 | 207.6 | 196.3 | 196.2 | 199.3 |
| (± S.E.M.) | (± 1.96) | (± 4.15) | (± 3.33) | (± 4.04) | (± 3.77) |

**TABLE 4.4-8. MEAN ORGAN WEIGHTS (g) AND ORGAN TO BODY WEIGHT RATIOS (g/100 g Body Wt) OF FEMALE RATS FOLLOWING 14 DAYS OF ORAL ADMINISTRATION OF 5.0 g/kg IRP LEACHATE SAMPLES**

| Organ | Sample L-3 | | Sample L-8 and L-10 | | |
|---|---|---|---|---|---|
| | Test | Control | L-8 Test | L-10 Test | Control |
| | n = 10 | n = 10 | n = 10 | n = 10 | n = 10 |
| Kidneys | 1.119 | 1.110 | 1.098 | 1.117 | 1.122 |
| (Ratio) | (0.77) | (0.76) | (0.79) | (0.81) | (0.79) |
| Heart | 0.574 | 0.556 | 0.514 | 0.549 | 0.532 |
| (Ratio) | (0.39) | (0.38) | (0.37) | (0.40) | (0.38) |
| Ovaries | 0.097 | 0.098 | 0.097 | 0.106 | 0.103 |
| (Ratio) | (0.07) | (0.07) | (0.07) | (0.08) | (0.07) |
| Brain | 1.734 | 1.713 | 1.726 | 1.722 | 1.719 |
| (Ratio) | (1.19) | (1.17) | (1.24) | (1.24) | (1.21) |
| Liver | 4.597 | 4.487 | 4.124 | 4.185 | 4.177 |
| (Ratio) | (3.15) | (3.05) | (2.96) | (3.02) | (2.94) |
| Spleen | 0.413 | 0.401 | 0.386 | 0.386 | 0.395 |
| (Ratio) | (0.28) | (0.27) | (0.28) | (0.28) | (0.28) |
| Thymus | 0.311 | 0.290 | 0.256 | 0.286 | 0.282 |
| (Ratio) | (0.21) | (0.20) | (0.18) | (0.21) | (0.20) |
| Lungs | 1.291 | 1.309 | 1.304 | 1.284 | 1.329 |
| (Ratio) | (0.89) | (0.89) | (0.94) | (0.93) | (0.94) |
| Adrenals | 0.069 | 0.070 | 0.065 | 0.066 | 0.080 |
| (Ratio) | (0.05) | (0.05) | (0.05) | (0.05) | (0.06) |
| Body Weight (g) | 145.9 | 147.1 | 139.2 | 138.7 | 141.9 |
| (± S.E.M.) | (± 2.62) | (± 1.15) | (± 0.89) | (± 1.63) | (± 1.57) |

Analysis of blood samples taken at sacrifice revealed no biologically significant differences between test and control groups.

*Subchronic Dermal Toxicity*

A total of 84 New Zealand White rabbits were dermally dosed for two weeks. Due to a limited quantity of the first sample, L-3, only six male and six female animals were included in the test and control groups. Ten males and ten females were used in each of the other test and control groups. One control group was used for both samples L-8 and L-10, which were received and tested concurrently. No toxic signs were noted in any of the 84 rabbits during the 14-day dosing period, and rabbits continued to gain weight during the test period (Table 4.4-9).

Organ weights were recorded at necropsy (Tables 4.4-10 and 4.4-11). The only statistically significant difference in organ weights and organ/body weight ratios between test and control groups was the larger testes in male rabbits exposed to sample L-8. Gross pathological examination of the animals at necropsy failed to reveal any dose-related lesions. Histopathologic examination of these tests has not been completed.

**TABLE 4.4-9. MEAN BODY WEIGHTS (g) OF NEW ZEALAND WHITE RABBITS IN THE SUBCHRONIC DERMAL TOXICITY STUDY**

| Test/Control Group (n = 6) | Male Rats | | | Female Rats | | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 14 | Day 0 | Day 7 | Day 14 |
| Control | 2.5 | 2.6 | 2.8 | 2.7 | 2.7 | 2.9 |
| (± S.E.M.) | (± 0.07) | (± 0.07) | (± 0.10) | (± 0.02) | (± 0.03) | (± 0.07) |
| L-3 | 2.5 | 2.5 | 2.7 | 2.7 | 2.7 | 2.9 |
| (± S.E.M.) | (± 0.03) | (± 0.05) | (± 0.05) | (± 0.07) | (± 0.04) | (± 0.04) |
| Control | 2.6 | 2.7 | 2.9 | 2.5 | 2.6 | 2.7 |
| (± S.E.M.) | (± 0.04) | (± 0.03) | (± 0.04) | (± 0.03) | (± 0.04) | (± 0.05) |
| L-8 | 2.6 | 2.6 | 2.8 | 2.6 | 2.7 | 2.8 |
| (± S.E.M.) | (± 0.03) | (± 0.03) | (± 0.05) | (± 0.04) | (± 0.04) | (± 0.05) |
| L-10 | 2.5 | 2.6 | 2.8 | 2.6 | 2.7 | 2.8 |
| (± S.E.M.) | (± 0.07) | (± 0.05) | (± 0.06) | (± 0.03) | (± 0.04) | (± 0.06) |

**TABLE 4.4-10. MEAN ORGAN WEIGHTS (g) AND ORGAN TO BODY WEIGHT RATIOS (g/100 g Body Wt) OF MALE RABBITS FOLLOWING 14 DAYS OF DERMAL EXPOSURE TO 2.0 g/kg IRP LEACHATE SAMPLES**

| Organ | Sample L-3 | | Sample L-8 and L-10 | | |
|---|---|---|---|---|---|
| | Test | Control | L-8 Test | L-10 Test | Control |
| | n = 6 | n = 6 | n = 9 | n = 10 | n = 10 |
| Liver | 91.017 | 101.250 | 104.711 | 107.900 | 110.830 |
| (Ratio) | (3.434) | (3.645) | (3.759) | (3.869) | (3.873) |
| Kidney | 18.250 | 19.217 | 17.778 | 19.160 | 19.620 |
| (Ratio) | (0.689) | (0.689) | (0.638) | (0.689) | (0.685) |
| Heart | 12.600 | 13.383 | 8.600 | 9.200 | 9.150 |
| (Ratio) | (0.477) | (0.479) | (0.310) | (0.334) | (0.319) |
| Brain | 8.717 | 9.083 | 8.922 | 9.030 | 9.020 |
| (Ratio) | (0.329) | (0.328) | (0.322) | (0.326) | (0.316) |
| Testes | 2.925 | 2.932 | 3.300[a] | 2.976 | 2.667 |
| (Ratio) | (0.111) | (0.104) | (0.119)[a] | (0.108) | (0.094) |
| Adrenals | 0.273 | 0.232 | 0.229 | 0.226 | 0.237 |
| (Ratio) | (0.010) | (0.008) | (0.008) | (0.008) | (0.008) |
| Thymus | 4.800 | 5.267 | 4.378 | 4.470 | 4.640 |
| (Ratio) | (0.182) | (0.189) | (0.156) | (0.161) | (0.162) |
| Spleen | 1.017 | 1.167 | 0.889 | 0.970 | 1.100 |
| (Ratio) | (0.039) | (0.041) | (0.032) | (0.035) | (0.039) |
| Lungs | 18.300 | 22.167 | 23.311 | 28.820 | 28.780 |
| (Ratio) | (0.690) | (0.805) | (0.841) | (1.028) | (1.014) |
| Body Weight (kg) (± S.E.M.) | 2.65 | 2.78 | 2.78 | 2.78 | 2.86 |
| | (± 0.050) | (± 0.098) | (± 0.047) | (± 0.061) | (± 0.040) |

[a] Significantly different from control at p<0.05.

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TABLE 4.4-11. MEAN ORGAN WEIGHTS (g) AND ORGAN TO BODY WEIGHT RATIOS (g/100 g Body Wt) OF FEMALE RABBITS FOLLOWING 14 DAYS OF DERMAL EXPOSURE TO 2.0 g/kg IRP LEACHATE SAMPLES

| Organ | Sample L-3 | | Sample L-8 and L-10 | | |
|---|---|---|---|---|---|
| | Test | Control | L-8 Test | L-10 Test | Control |
| | n = 6 | n = 6 | n = 10 | n = 10 | n = 10 |
| Liver | 97.350 | 97.817 | 107.190 | 106.400 | 96.890 |
| (Ratio) | (3.330) | (3.386) | (3.764) | (3.746) | (3.536) |
| Kidney | 18.700 | 17.783 | 18.860 | 18.250 | 18.590 |
| (Ratio) | (0.641) | (0.616) | (0.664) | (0.645) | (0.678) |
| Heart | 13.500 | 13.650 | 8.530 | 8.620 | 8.810 |
| (Ratio) | (0.462) | (0.471) | (0.335) | (0.306) | (0.323) |
| Brain | 8.933 | 8.917 | 8.610 | 8.840 | 8.700 |
| (Ratio) | (0.307) | (0.310) | (0.303) | (0.314) | (0.318) |
| Ovaries | 0.210 | 0.235 | 0.221 | 0.230 | 0.192 |
| (Ratio) | (0.007) | (0.008) | (0.008) | (0.008) | (0.007) |
| Adrenals | 0.282 | 0.247 | 0.204 | 0.210 | 0.204 |
| (Ratio) | (0.010) | (0.009) | (0.007) | (0.007) | (0.007) |
| Thymus | 4.500 | 4.717 | 4.440 | 4.370 | 4.080 |
| (Ratio) | (0.154) | (0.164) | (0.157) | (0.154) | (0.150) |
| Spleen | 0.933 | 1.083 | 1.160 | 1.420 | 1.190 |
| (Ratio) | (0.032) | (0.038) | (0.041) | (0.050) | (0.043) |
| Lungs | 23.433 | 24.600 | 27.650 | 30.210 | 26.250 |
| (Ratio) | (0.802) | (0.855) | (0.971) | (1.069) | (0.959) |
| Body Weight (kg) ( ± S.E.M.) | 2.92 (±0.040) | 2.88 (±0.070) | 2.84 (±0.045) | 2.83 (±0.056) | 2.74 (±0.045) |

Analysis of blood samples taken at sacrifice revealed no biologically significant differences between test and control groups. In addition, no biologically significant differences were revealed in comparisons of pre- and post-test blood data within groups.

## DISCUSSION

The three leachate samples presented for testing to date have not demonstrated any acute or subchronic oral toxicity, skin irritation, or sensitization potential. Weight losses were observed in the subchronic oral toxicity study in both male and female test and control groups during the first week of testing, but this is believed to be a response to the overnight fast prior to dosing each day.

The IRP samples did not demonstrate any subchronic dermal toxicity to female rabbits and only sample L-8 showed possible toxicity to male rabbits. The testes of male rabbits dermally exposed for 14 days to sample L-8 were significantly heavier at sacrifice than those of control rabbits (p<0.05). Gross pathological examination did not reveal any abnormalities; histopathologic examination of these tissues has not been completed.

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Mild eye irritation was present following instillation of each of the three IRP samples; however, the irritation typically was resolved or was in the process of resolving by 72 h post-treatment. Conjunctival redness and corneal abrasions were the forms of irritation observed, and both were attributed to abrasion from particles in the samples. Each sample contained particulate material on receipt, and despite refrigeration and continuous stirring, the particle content of each container appeared to increase with increasing storage time.

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## APPENDIX 1

### EVALUATIONS AND SCORING OF SKIN REACTIONS

| Parameter | Score |
|---|---|
| 1. Erythema | |
| No erythema | 0 |
| Very slight erythema (barely perceptible) | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema (beet redness) | 4 |
| | |
| 2. Edema | |
| No edema | 0 |
| Very slight edema (barely perceptible) | 1 |
| Slight edema (edges of area well-defined by definite raising) | 2 |
| Moderate edema (raising approx. 1mm) | 3 |
| Severe edema (raising more than 1 mm and extending beyond area of exposure) | 4 |
| | |
| 3. Necrosis[a] | |
| No necrosis | 0 |
| Slight necrosis (less than one-fourth of the exposed area) | 5 |
| Moderate necrosis (one-fourth to one-half of the exposed area) | 10 |
| Severe necrosis (more than one-half of the exposed area) | 15 |

[a] Necrosis, for the purpose of this scoring system, is defined as a chemical denaturation of tissue sufficiently severe to result in fibrotic replacment (scar tissue). Superficial eschar, which heals without scarring, is not classified as necrosis.

## APPENDIX 2

## DRAIZE[a] SCALE FOR SCORING OCULAR LESIONS

| Parameter | Score |
|---|---|
| **1. Cornea** | |
| A. Opacity-degree of density (area most taken for reading) | |
| No opacity | 0 |
| Scattered or diffuse area, details of iris clearly visible | 1 |
| Easily discernible translucent areas, details of iris slightly obscured | 2 |
| Opalescent areas, no details of iris visible, size of pupil barely discernible | 3 |
| Opaque, iris invisible | 4 |
| B. Area of cornea involved | 0 |
| One-quarter (or less), but not zero | 1 |
| Greater than one-quarter, but less than one-half | 2 |
| Greater than one-half, but less than three-quarters | 3 |
| Greater than three-quarters, up to whole area | 4 |
| Score = A x B x 5　　　　　　Total Maximum = | 80 |
| **2. Iris** | |
| A. Values | |
| Normal | 0 |
| Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or a combination of any thereof) iris still reacting to light (sluggish reaction is positive) | 1 |
| No reaction to light, hemorrhage, gross destruction (any or all of these) | 2 |
| Score = A x 5　　　　　　Total Maximum = | 10 |
| **3. Conjuctivae** | |
| A. Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris) | |
| Vessels normal | 0 |
| Vessels definitely injected above normal | 1 |
| More diffuse, deeper crimson red, individual vessels not easily discernible | 2 |
| Diffuse beefy red | 3 |
| B. Chemosis | |
| No swelling | 0 |
| Any swelling above normal (included nictitating membrane) | 1 |
| Obvious swelling with partial eversion of lids | 2 |
| Swelling with lids about half closed | 3 |
| Swelling with lids about half closed to completely closed | 4 |
| C. Discharge | |
| No discharge | 0 |
| Any amount different from normal (does not include small amounts observed in inner canthus of normal animals) | 1 |
| Discharge with moistening of the lids and hairs just adjacent to lids | 2 |
| Discharge with moistening of the lids and hairs, and considerable area around the eye | 3 |
| Score = (A + B + C) x 2　　　　　　Total Maximum = | 20 |

The MAXIMUM TOTAL SCORE is the sum of all scores obtained
for the cornea, iris, and conjunctivae.

Total Maximum Score Possible = 110

[a] Draize et al. (1944.)

## APPENDIX 3

## GRADING OF SKIN REACTIONS IN THE MAGUIRE GUINEA PIGS SENSITIZATION TEST

| Erythema | Score | Edema | Score |
|---|---|---|---|
| None | 0 | None | 0 |
| Very slight pink | 1 | Very slight | 1 |
| Slight pink | 2 | Slight | 2 |
| Moderate red | 3 | Moderate | 3 |
| Very red | 4 | Marked | 4 |

## APPENDIX 4

## SCALE FOR SENSITIZATION CLASSIFICATION

| Positive Responders (%) | Sensitization Potential |
|---|---|
| None | None |
| Very slight pink | Very slight |
| Slight pink | Slight |
| Moderate red | Moderate |
| Very red | Marked |

# SECTION 5

## STUDIES ON AIR FORCE FUELS

### 5.1 URINARY METABOLITE IDENTIFICATION AND SYNTHESIS OF HYDROCARBONS

M. Serve[a] and R. Conolly

**INTRODUCTION**

Some hydrocarbon fuels used by the Air Force cause a nephropathy in male rats. The active compounds in these fuels include bicyclic hydrocarbons, such as *cis*- and *trans*-decalin and tetralin, as well as acyclic branched chain hydrocarbons, such as 2,2,4-trimethylpentane and 2,3,4-trimethyl-pentane. The monocyclic hydrocarbon cyclohexane was shown to be non-nephrotoxic. Alkyl substituted cyclohexanes, many of which are found in various fuel and solvent mixtures, have not been examined for their ability to induce nephrotoxic effects in male rats. Thus, methylcyclohexane, isopropylcyclohexane, and *t*-butylcyclohexane are being examined individually to evaluate their nephrotoxic potential in male rats.

**RESEARCH PLAN**

The overall research effort directed toward this problem consists of nephrotoxicity evaluation and metabolism studies. Twelve-week-old male Fischer 344 rats will be orally gavaged with one of the three related hydrocarbons, observed daily, and urine will be collected 48-h post-exposure. Gross and histological examinations will be performed. For the metabolism studies, hydrocarbon metabolites extracted from urine and kidneys will be identified and synthesized. Major metabolites will be administered to new groups of male rats to test their ability to induce hyaline droplet nephropathy and ascertain any specific metabolic and/or structure activity relationships involved.

**PROCEDURE**

Groups of seven male rats were dosed with the individual hydrocarbons (0.8 g/kg) on an every-other-day regimen for 14 days. Urine was collected for the first 48-h dosing period and prepared for analysis. After 14 days, the rats were sacrificed. One kidney was submitted for pathologic examination, while the other kidney was homogenized and extracted to isolate hydrocarbon metabolites. Control rats were dosed with water and handled in the same manner as the hydrocarbon-treated animals.

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## RESULTS

This is a collaborative project between the THRU and the Air Force with the THRU responsible for the metabolism studies. For t-butylcyclohexane, the chemicals listed in Table 5.1-1 were synthesized, and their GC retention times and mass spectrometry fragmentation patterns were compared to the urinary metabolites.

The materials will be provided to the Air Force for toxicity evaluation as described above.

#### TABLE 5.1-1. POTENTIAL METABOLITES OF t-BUTYLCYCLOHEXANE THAT HAVE BEEN SYNTHESIZED AND COMPARED TO CHEMICALS FOUND IN THE URINE OF RATS TREATED WITH THE PARENT COMPOUND

2-t-butylcyclohexanol (cis- and trans-)

3-t-butylcyclohexanol (cis- and trans-)

2-t-butylcyclohexanone

3-t-butylcyclohexanone

2-methyl-2-cyclohexylpropanoic acid (F)

2-methyl-2-cyclohexylpropanol (F)

2-cis-hydroxy-4-trans-t-butylcyclohexanol (F)

2-cis-hydroxy-4-cis-t-butylcyclohexanol (F)

2-trans-hydroxy-4-trans-t-butylcyclohexanol (F)

2-trans-hydroxy-4-cis-t-butylcyclohexanol (F)

2-methyl-2-cyclohexyl-1,3-propanediol

F = found in rat urine

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## 5.2. *IN VITRO* EFFECTS OF SOLUBILIZED 2,3,4-TRIMETHYLPENTANE USING PRIMARY RAT HEPATOCYTES

### N. De.Raso and C. Flemming

### INTRODUCTION

Studies conducted at the Armstrong Aerospace Medical Research Laboratory (AAMRL) have shown sex differences in the response of rats exposed to the vapors of hydrocarbon propellants such as JP-4 and JP-5 (complex mixtures of aliphatic and aromatic hydrocarbons; Bruner and Pitts, 1983). Kidney lesions occur only in male rats after exposure to hydrocarbon propellant vapors. These lesions consist of greatly increased cytoplasmic hyaline droplets in the epithelial cells of the proximal tubule, necrosis of proximal tubular cells, and intratubular blockage by cell debris at the junction of the outer and inner stripe of the outer medulla (Bruner, 1984). Long-term exposure of male rats to these hydrocarbon vapors results in abundant mineralized casts in the medullary tubules and a significant increase in the incidence of renal tumors (Bruner, 1984). Studies investigating the toxic and carcinogenic effects of "pure" hydrocarbon propellants such as 2,3,4-trimethylpentane (TMP), 2,2,4-TMP, RJ-4 propellant and JP-10, in most cases, have resulted in similar kidney lesions in male rats as those produced by hydrocarbon mixtures (Bruner and Pitts, 1983). To assess better the risks to Air Force personnel working with hydrocarbon propellants, the pathogenesis of hydrocarbon nephropathy needs to be characterized. 2μ globulin

In an effort to characterize the pathogenesis of hydrocarbon propellants, TMP, a principal constituent of gasoline involved in assigning "octane rating," has been used in gavage studies. A single gavage dose of TMP was found to produce hydrocarbon nephropathy in male Fischer 344 rats (Hobson *et al.*, 1985). The major products of 2,2,4-TMP metabolism by the kidneys of male rats have been identified in urine (Olson *et al.*, 1985), and a theoretical metabolic pathway has been described. However, the metabolism by the liver has not yet been investigated. The production of alpha 2μ-globulin, a low molecular weight (MW 26,400) protein, in the liver of male rats is influenced by testosterone and other interactive hormones (Irwin *et al.*, 1971). This protein is filtered by the kidney through the glomeruli and is reported to be the major urinary protein in male rats. Alpha 2μ-globulin is not synthesized appreciably by the liver of normal female rats or other species (Roy, 1973). Therefore, alpha 2μ-globulin is believed to be the major constituent of the cytoplasmic hyaline droplets seen in epithelial cells of the proximal tubule of male rats exposed to hydrocarbon vapors, and evidence for this has been reported (Alden *et al.*, 1983). Factors responsible for the excessive accumulation of alpha 2μ-globulin in the proximal tubular cells may be responsible for the pathogenesis of hydrocarbon nephropathy. It is possible that TMP and/or its metabolites inhibit

proximal tubular lysosomal enzymes, or other catabolic protein, preventing the degradation and excretion of alpha 2μ-globulin.

The objective of this study was to isolate and establish primary cultures of male rat hepatic cells suitable for experimental exposure to sublethal concentrations of TMP. The viability of isolated cells was measured to determine satisfactory isolation procedures. Using cell lysis as a measure of toxicity, concentration-response curves were determined for TMP to establish suitable treatment concentrations for metabolic studies. The appropriate exposure of liver cells to TMP for metabolic studies was implemented. and the samples were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

## MATERIALS AND METHODS

### Liver Perfusion and Hepatocyte Isolation

Livers were perfused with two different media for blood removal and digestion, respectively. Blood removal was accomplished by perfusing the liver with a modified Hank's balanced salt solution (HBSS) (Gibco; calcium and magnesium free, without phenol red) supplemented with bovine serum albumin (BSA; 500 μg/mL; Fraction V), heparin (2.0 U/mL), insulin (0.5 μg/mL), and sodium bicarbonate (2.2 mg/mL). This perfusion was followed, without interruption of flow, by a second modified HBSS (Gibco; without phenol red) supplemented with BSA (500 μg/mL), insulin (0.5 μg/mL), sodium bicarbonate (2.2 mg/mL), and collagenase (Sigma Type IV; 0.5 mg/mL 540 U/mg).

Primary hepatocytes were isolated using the procedures of Berry and Friend (1969), as described by Bonney (1974) and Oldam et al. (1979), with the following modifications. Livers were perfused using a 23-gauge butterfly infusion set secured with sutures and a bulldog clamp. After perfusing the liver with the two perfusion media in tandem, at a flow rate of 35 to 40 mL/min, the liver was removed to a sterile petri dish containing the HBSS digestion medium, and single cells were released by gentle combing with a ~50-mm dog comb.

### Hepatocyte Enrichment and Culture

Leibovitz L-15 tissue culture medium without albumin (L-15A) was supplemented as described by Kreamer (1985), and modified by the addition of Sigma Media Supplement (10 ml/L results in a final concentration of 5.0 μg insulin/mL, 5.0 μg transferrin/mL, and 5.0 μg sodium selenite/mL), which was added aseptically to sterile L-15A the day the medium was to be used (pH 7.2 to 7.4).

A modified HBSS medium for washing primary hepatocytes (HBSSA) was prepared according to the method of Kreamer (1986), except that BSA (0.5 g/L) was added (pH 7.2 to 7.4).

Primary hepatocytes were enriched using low-speed, isodensity Percoll (Pharmacia) centrifugation as described by Kreamer (1986). Isolated hepatocytes were washed 2X with HBSSA

and were then resuspended to a desired concentration (ranging from ~$3 \times 10^5$ to $1 \times 10^6$ cells/m$^{-1}$) in L-15A culture medium. Four milliliters of the resultant cell suspension were then added to appropriately labeled sterile 25-mL Erlenmyer flasks (Wheaton) and incubated at 37°C (95% oxygen: 5% carbon dioxide) for 4 h on a clinical rotator at 90 rpm.

### Determination of $LC_{50}$

The $LC_{50}$ for liver cells using TMP (Wiley Organics) was determined by exposing hepatocytes to selected concentrations of TMP, ranging from 3.15 mM to 31.5 mM. The TMP was solubilized 1:1 by volume in acetone, and subsequent TMP dilutions were made in acetone such that the final concentration of acetone in the medium remained constant at 0.5%, while the TMP concentration varied. Both a negative control and a 0.5% acetone (vehicle) control were also run. Duplicate flasks of exposed and control liver cells were incubated for 4 h. The percent viability was determined for all control and treated cells by trypan blue-exclusion hemacytometer cell counts. Based on the $LC_{50}$, a sublethal concentration of TMP (12 mM) was chosen to expose liver cells for metabolite determination.

### Lactate Dehydrogenase (LDH) Assay

LDH leakage from control and TMP-exposed cells were determined from the hepatocyte cell suspension cultures used in the $LC_{50}$ determination experiments. One-half milliliter samples from each flask, in all control and treatment groups, were removed at 0 and 4-h time periods and placed into appropriately labeled 2-mL-capped centrifuge tubes, and centrifuged at 7000 $g$ for 5 min. After centrifugation, 0.4 mL of the supernatant from each 2-mL-tube was removed and added to a second set of appropriately labeled 2-mL-capped centrifuge tubes. These samples were stored at 4°C until assayed 24 h later. Samples were diluted 1:4 (v/v) with distilled water and then assayed by a Du Pont ACA V discrete clinical analyzer for LDH activity (Du Pont Co.).

### Metabolite Determination

Duplicate flasks of liver cells, at a cell density of $4 \times 10^6$ cells/mL, were exposed to 12 mM TMP for 4 h. At the end of the exposure, the percent viability was determined. The cell suspensions were then centrifuged and the supernatants and pelleted cells were saved for extraction of metabolites. Samples were analyzed by GC and GC/MS.

### Statistical Analysis

Probit analysis was used to calculate the $LC_{50}$ with a 95% confidence interval (Finney, 1971). The procedure was performed using a SAS procedure called Probit. Since acetone, the control vehicle for TMP, was found to have a lethality rate greater than zero, a C factor was used. The C factor, the chance of cell death resulting from acetone treatment, was calculated as follows:

$$C = \frac{(\text{experiment 1 \# of acetone indexed dead cells} + \text{experiment 2 \# of acetone induced dead cells})}{(\text{experiment 1 initial cell seeding} + \text{experiment 2 initial cell seeding})}$$

The number of dead cells resulting from acetone treatment was found by subtracting the zero time period from the 4-h time period.

## RESULTS

### $LC_{50}$ Determination

Using primary hepatocyte cell suspension cultures of ~$5 \times 10^5$ cells/mL, solubilized TMP was tested at selected concentrations from 3.15 mM to 31.5 mM to determine the $LC_{50}$ in medium without albumin. Table 5.2-1 indicates that the $LC_{50}$ for TMP is ~15.7 mM. When this experiment was repeated using primary hepatocyte cell suspension cultures of ~$7 \times 10^5$ cells/mL, the $LC_{50}$ for TMP was again found to be ~15.7 mM (Table 5.2-2). It was noted that 15 to 32% of the primary hepatocytes in both control groups were not viable after the 4-h incubation period for the experiment. When the data from these two experiments were combined and analyzed by SAS Probit analysis, the $LC_{50}$ was calculated to be 14.6 mM TMP.

The above TMP experiment, which was used to establish the *in vitro* $LC_{50}$ for TMP, was repeated with 0.5% albumin incorporated into the cell suspension medium. Using primary hepatocyte suspension cultures of ~$3 \times 10^5$ cells/mL, the $LC_{50}$ for TMP was between 15.7 and 31.5 mM (Table 5.2-3). When this experiment was repeated using cultures at a cell density of ~$2 \times 10^5$ cells/mL, similar results were obtained (Table 5.2-4). In general, 27 to 38% of the primary hepatocytes in both control and vehicle groups were not viable after the 4-h incubation period used for the experiment. When the data from these two experiments were combined and analyzed by SAS Probit analysis, the $LC_{50}$ for TMP was calculated as 17.0 mM.

### LDH Leakage

LDH leakage was measured along with viability when establishing the *in vitro* $LC_{50}$ for TMP to determine how well LDH leakage from the cells correlated with the $LC_{50}$ data. Total lysis of the primary hepatocytes in medium without albumin occurred at 31.5 mM TMP and resulted in the release of ~2.4 U/mL of LDH. Figure 5.2-1 indicates that at the approximate $LC_{50}$ for TMP in medium without albumin (~15.7 mM), a ~2.5-fold increase in LDH release occurred when compared with the two control groups. The LDH data from the $LC_{50}$ experiment using a cell density of ~$7 \times 10^5$ cells/mL indicated that an ~1.5-fold increase in LDH leakage occurred at the $LC_{50}$ when compared to the two control groups (Figure 5.2-2). SAS Probit anaysis of the combined data from these two experiments

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indicated that a twofold increase in LDH leakage from control levels occurred at 15.7 mM TMP. This indicates that at the $LC_{50}$ for TMP, ~50% of the total cellular LDH has leaked into the medium.

TABLE 5.2-1. VIABILITY OF PRIMARY HEPATOCYTE SUSPENSION CULTURES (~5 x $10^5$ CELLS/ML) EXPOSED TO SOLUBILIZED TMP FOR FOUR HOURS IN MEDIUM WITHOUT ALBUMIN

| Treatment | Cell Count[a] | % of Control[b] |
|---|---|---|
| Control | $3.3 \times 10^5$ | - |
| Vehicle[c] | $3.4 \times 10^5$ | - |
| 3.15 mM TMP | $3.2 \times 10^5$ | 94 |
| 7.90 mM TMP | $3.3 \times 10^5$ | 97 |
| 15.7 mM TMP | $1.5 \times 10^5$ | 44 |
| 31.5 mM TMP | 0 | 0 |

[a] Mean of three counts. Number of cells/milliliter determined by trypan blue exclusion hemacytometer counts.
[b] The control value used to compare the experimental exposure groups was derived from the average of the control and vehicle control groups from duplicate flasks, respectively, and was $3.4 \times 10^5$ cells/mL.
[c] The vehicle control consisted of primary hepatocyte suspension cultures treated with a 1:1 dilution of sterile acetone and distilled water to a final concentration of 0.5%.

TABLE 5.2-2. VIABILITY OF PRIMARY HEPATOCYTE SUSPENSION CULTURES (~7 x $10^5$ CELLS/ML) EXPOSED TO SOLUBILIZED TMP FOR FOUR HOURS[a] IN MEDIUM WITHOUT ALBUMIN

| Treatment | Cell Count | % of Control[b] |
|---|---|---|
| Control | $6 \times 10^5$ | - |
| Vehicle | $6 \times 10^5$ | - |
| 7.90 mM TMP | $5.4 \times 10^5$ | 90 |
| 12.0 mM TMP | $4 \times 10^5$ | 67 |
| 15.7 mM TMP | $3.2 \times 10^5$ | 53 |
| 31.5 mM TMP | 0 | 0 |

[a] Conditions were identical to Table 5.2-1.
[b] The control value used to compare the experimental exposure groups was derived from the average of the control and vehicle control groups from duplicate flasks, respectively, and was $6 \times 10^5$ cells/mL.

**TABLE 5.2-3. VIABILITY OF PRIMARY HEPATOCYTE SUSPENSION CULTURES ($\sim 5 \times 10^5$ CELLS/ML) EXPOSED TO SOLUBILIZED TMP FOR FOUR HOURS IN MEDIUM CONTAINING 0.5% ALBUMIN[a]**

| Treatment | Cell Count | % of Control[b] |
|---|---|---|
| Control | $2.1 \times 10^5$ | - |
| Vehicle | $2.4 \times 10^5$ | - |
| 3.15 mM TMP | $2.6 \times 10^5$ | 118 |
| 7.90 mM TMP | $2.3 \times 10^5$ | 104 |
| 15.7 mM TMP | $2.2 \times 10^5$ | 100 |
| 31.5 mM TMP | $2.0 \times 10^4$ | 9 |

a  Conditions for this experiment were identical to that stated in Table 5.2-1, except that albumin was incorporated into the medium.
b  The control value used to compare the experimental exposure groups was derived from the average of the control and vehicle groups from duplicate flasks, respectively, and was $2.2 \times 10^5$ cells/mL.


**TABLE 5.2-4. VIABILITY OF PRIMARY HEPATOCYTE SUSPENSION CULTURES ($\sim 2 \times 10^5$ CELLS/ML) EXPOSED TO SOLUBILIZED TMP FOR FOUR HOURS IN MEDIUM CONTAINING 0.5% ALBUMIN[a].**

| Treatment | Cell Count | % of Control[b] |
|---|---|---|
| Control | $1.2 \times 10^5$ | - |
| Vehicle | $1.3 \times 10^5$ | - |
| 3.15 mM TMP | $1.3 \times 10^5$ | 108 |
| 7.90 mM TMP | $1.2 \times 10^5$ | 100 |
| 15.7 mM TMP | $9.0 \times 10^4$ | 75 |
| 31.5 mM TMP | 0 | 0 |

a  Conditions identical to those in Table 5.2-3.
b  The control value used to compare the experimental exposure groups was derived from the average of the control and vehicle control groups from triplicate flasks, respectively, and was $1.2 \times 10^5$ cells/mL.

LDH
(U/L)

2,400 —

1,800 —

1,200 —

600 —

0 —

|  | Control | Vehicle[b] | 3.15 | 7.9 | 15.7 | 31.5 | Control | Vehicle[b] | 3.15 | 7.9 | 15.7 | 31.5 |

(360)[a]  (285)  (358)  (295)  (298)  (445)          (482)  (568)  (595)  (635)  (1298)  (2368)

mM TMP                                        mM TMP

0 Hours                                        4 Hours

[a]  The number in parentheses indicates the average LDH leakage (U/L) from duplicate flasks.
[b]  Vehicle consisted of dosing hepatocyte cultures with a sterile 0.5% acetone solution.

Figure 5.2-1. LDH Leakage from Primary Hepatocyte Suspension Cultures (~ 5 x 10⁵ cells /mL) Exposed to TMP for Four Hours in Medium Without Albumin.

Figure 5.2-2. LDH Leakage from Primary Hepatocyte Suspension Cultures (~ $7 \times 10^5$ cells /mL) Exposed to TMP for Four Hours in Medium Without Albumin.

LDH leakage from primary hepatocyte suspension cultures incorporating 0.5% albumin during TMP exposure resulted in decreased LDH leakage from treated cells. Treatment of primary hepatocytes with 15.7 mM TMP ($LC_{50}$ in medium lacking albumin) at a cell density of ~3 x $10^5$ cells/mL in medium containing albumin, resulted in LDH release similar to that observed in the control groups (Figure 5.2-3). A 50% increase in LDH release was found to occur at 31.5 mM TMP; a twofold increase in the concentration of TMP to effect a comparable LDH release to that seen in medium lacking albumin (Figure 5.2-3). Although the second TMP experiment in medium containing albumin at a cell density of ~2 x $10^5$ cells/mL appeared to produce similar results as those seen in the first experiment at a cell density of 3 x $10^5$ cells/mL, it appears that the 50% increase in LDH release seen at 31.5 mM TMP in the first experiment did not occur in the second experiment (Figure 5.2-4). When the data from both of these experiments were combined and analyzed by SAS Probit analysis, it was calculated that the concentration of TMP corresponding to a 50% increase in LDH release as compared to control levels, was 27.7 mM. These data support the previous SAS Probit analysis of the viability data, when the data from the 31.5-mM TMP group are not included for exposures in medium containing albumin, which gave a calculated $LC_{50}$ value of 31.2 mM TMP. This finding indicates that LDH and viability data are in agreement. Therefore, the concentration of TMP required to effect a 50% increase in LDH leakage over control levels in medium containing 0.5% albumin appears to have increased approximately twofold over that seen in medium lacking albumin.

### Metabolite Determination

Metabolites from TMP metabolism by 4-h primary hepatocyte suspension cultures at a cell density of ~2 x $10^6$ cells/mL or less, could not be detected in either the culture medium or the cell homogenate. When a cell density of ~4 x $10^6$ cells/mL was used, a metabolite, 2,3,4-trimethyl-1-pentanoic acid (TMPA), was detected in the culture medium. This metabolite had a GC/MS retention time of 14.6 min and correlated very well with a previously determined TMPA GC retention time of 14.1 min under the same conditions (Yu et al., 1987). A recently published GC/MS tracing of TMPA (Yu et al., 1987; Figure 5.2-5a) also agreed with the GC/MS tracing for the metabolite extracted from the hepatocyte culture medium (Figure 5.2-5b). No metabolite was detected in the cell homogenate.
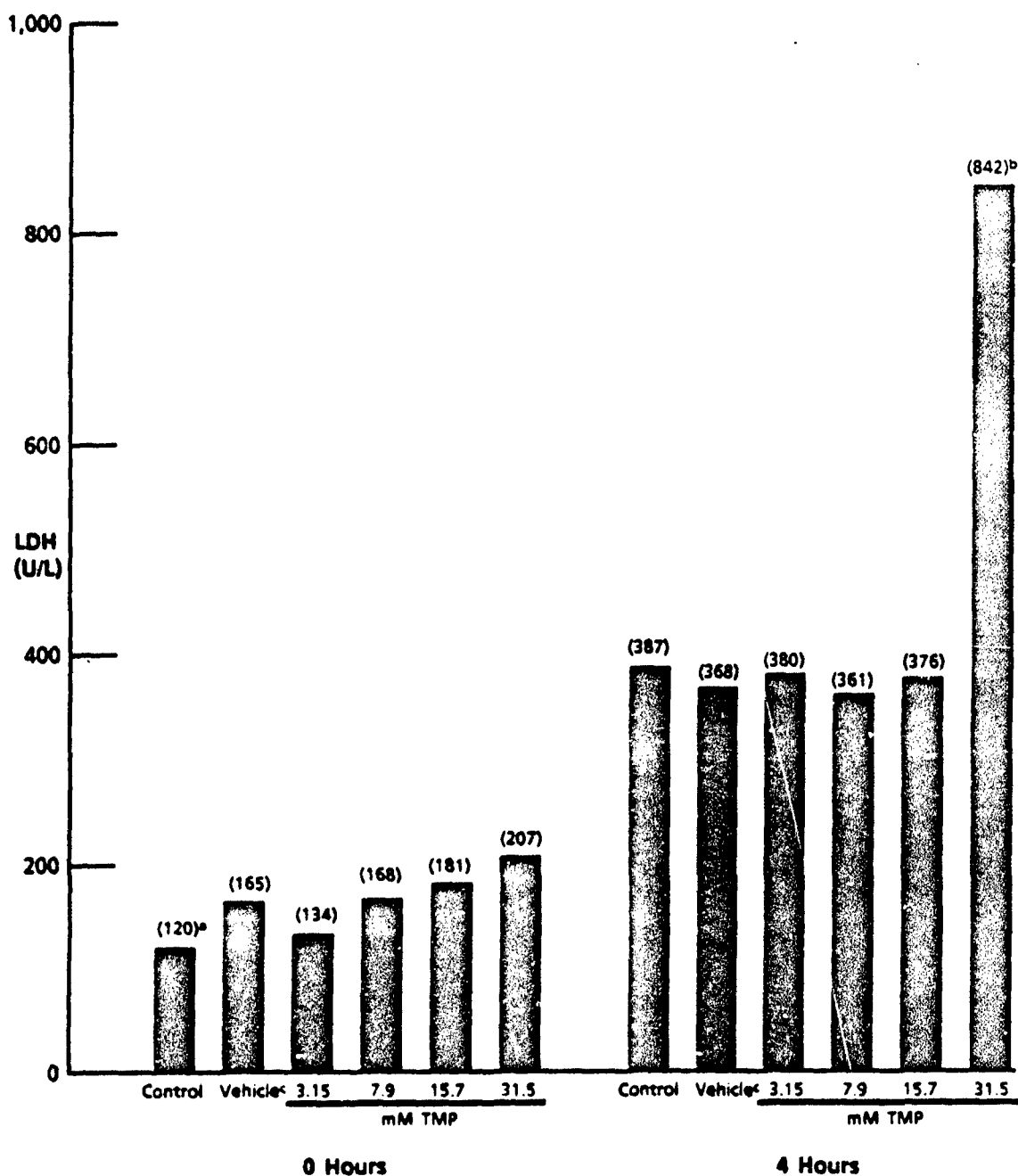
- a    The number in parentheses indicates the average LDH leakage (U/L) from duplicate flasks.
- b    Value from one flask.
- c    Vehicle consisted of dosing hepatocyte cultures with a sterile 0.5% acetone solution.

Figure 5.2-3.  LDH Leakage from Primary Hepatocyte Suspension Cultures (~ 3 x 10^5 cells /mL)
Exposed to TMP for Four Hours in Medium Containing 0.5% Albumin.

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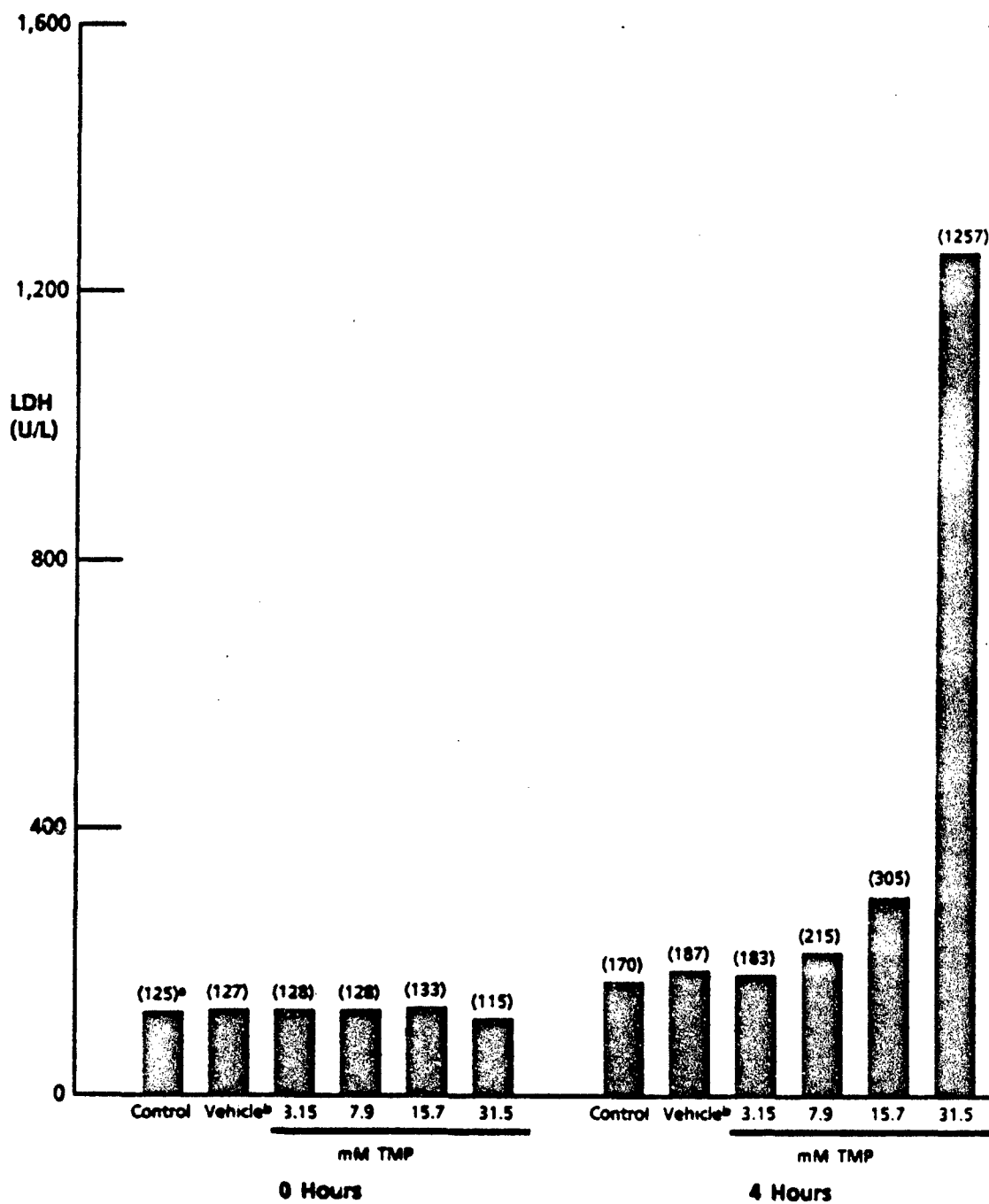
- a The number in parentheses indicates the average LDH leakage (U/L) from duplicate flasks.
- b Vehicle consisted of dosing hepatocyte cultures with a sterile 0.5% acetone solution.

Figure 5.2-4. LDH Leakage from Primary Hepatocyte Suspension Cultures (~ 2 x 10^{5} cells /mL) Exposed to TMP for Four Hours in Medium Containing 0.5% Albumin.

Figure 5.2-5. Mass Spectra of (a) 2,3,4-Trimethyl-1-pentanoic Acid from Yu et al. (1987), (b) Extract from Primary Hepatocyte Suspension Culture Medium.

## DISCUSSION

To establish the *in vitro* $LC_{50}$ for solubilized TMP, the environmental culture conditons played an important role in determining the toxicity of TMP. When TMP experiments were implemented using primary hepatocyte suspension cultures in L-15A medium without albumin, the $LC_{50}$ was found to be 14.6 mM when analyzed by the SAS Probit procedure. When these experiments were 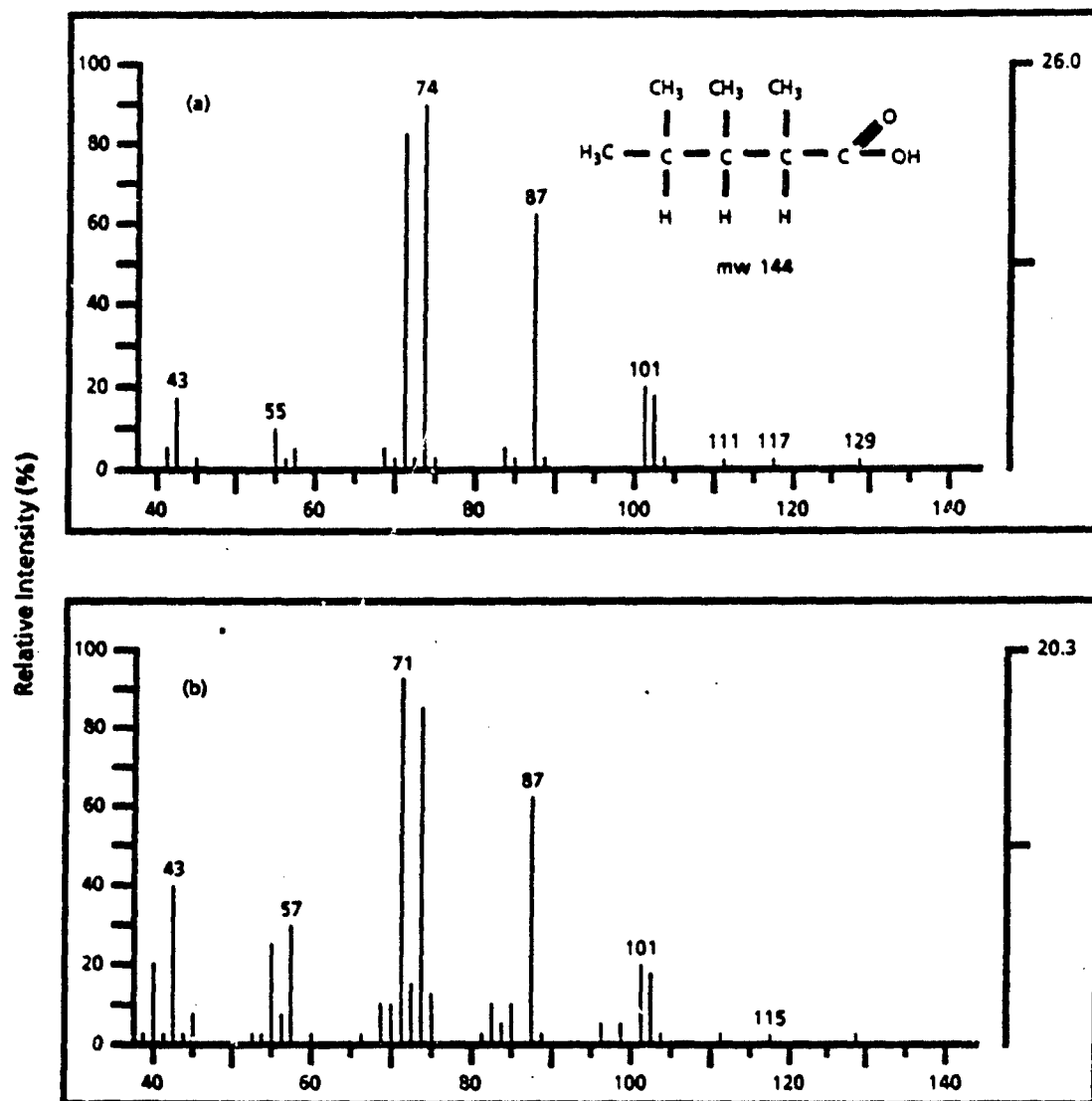repeated in medium containing 0.5% albumin, an increase in the $LC_{50}$ concentration of TMP (17.0 mM) was calculated by SAS Probit analysis. This increase was underestimated because total lysis of the hepatocytes had occurred at the 31.5-mM concentration, which did not occur in the first experiment. When the data from this group in both experiments were not used in the calculation, a $LC_{50}$ of 31.2 mM TMP was indicated. A confidence interval could not be established for this $LC_{50}$ calculation, because none of the TMP treatment groups used for this calculation were found to differ significantly from the control groups. Therefore, an accurate "good fit" of the data could not be determined. However, the calculated $LC_{50}$ value of 31.2 mM TMP correlated well with the LDH data from these two experiments, which indicated that a 50% increase in LDH leakage over control values occurs at 27.7 mM TMP.

The calculated TMP concentration (27.7 mM) that would have resulted in a 50% increase in LDH release in medium containing albumin may actually be greater, because the total amount of LDH had been released (due to total cell lysis) in the 31.5 mM TMP treatment group of the second experiment. This did not occur in the first experiment. It is possible that the observations of the second experiment at the 31.5-mM TMP concentration resulted from cellular instability due to low initial seeding.

The calculated $LC_{50}$ value of 31.2 mM TMP in medium containing albumin appears reasonable, since at 15.7 mM TMP the average of the percent-of-control cells surviving in these two experiments (88%) had nearly doubled from that seen in the two experiments in medium without albumin (48%). Therefore, some TMP is probably being bound in media containing albumin. Additional experiments will be implemented to verify these findings.

Metabolites of 2,2,4-TMP have been identified in the urine of male rats (Olson et al., 1985), and recently the metabolites of TMP have also been identified (Yu et al., 1987). The metabolites of TMP identified in the urine of male rats are 2,3,4-trimethyl-1-pentanol, 2,3,4-trimethyl-2-pentanol, and TMPA. In this study, the metabolite TMPA was detected in the medium of hepatocyte suspension cultures treated with 12 mM TMP. This is the first study to investigate solubilized TMP by *in vitro* methods. These data also provide direct evidence that the metabolite TMPA is produced by the liver and excreted by the kidney. Additional samples will be tested to characterize further and verify the metabolites of TMP metabolism by the liver.

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## 5.3 PHYSICAL CHARACTERIZATION AND *IN VIVO* CLEARANCE OF BERYLLIUM ROCKET EXHAUST PARTICLES

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### INTRODUCTION

United States Air Force research and development efforts in rocket propellants has stimulated renewed interest in the toxic potential of exhaust from rockets fueled with beryllium (Be-) bearing fuels. Although much is already known about the toxicity of Be and its oxides, it is currently unclear how this information is related to the material produced under the combustion conditions inside a rocket motor. In general, ingestion and inhalation are considered to be the major routes of exposure for humans and animals. Oral exposure to environmentally relevant levels of Be compounds has not been shown to produce significant toxicity (Callahan, 1979). However, both acute and chronic toxic effects are recognized in both animals and humans following inhalation of various forms of Be, including Beryllium oxide (BeO), from occupational exposure or from laboratory experiments. Acute inhalation of aerosols having a high soluble Be content leads to inflammation of the respiratory tract and pulmonary edema (Wilbur, 1980). A few cases of acute pneumonitis following repeated human exposure to low levels of Be have been reported. Berylliosis was first described in fluorescent lamp workers in the 1940s (Hardy and Tabershaw, 1946). This disease is a chronic immunologically mediated disease which results in a granulomatous hypersensitivity response in the lung. Pathogenesis of this disease typically follows a prolonged time course, up to 25 years, with progressive pulmonary insufficiency as the principle manifestation. Beryllium carcinogenicity is well established in several species of laboratory animals, but the human cancer risk is not well established (Wagoner et al., 1980).

The severity of BeO toxicity apparently is a function of the temperature at which the BeO is formed. BeO prepared at low temperatures (less than 500°C) appears to be more toxic and carcinogenic than material produced at 1600°C (Spencer et al., 1968, 1972). It has been suggested that differences in solubility may account for this difference in toxicity.

To determine whether rocket motor exhaust particles are similar to low or high temperature fired BeO, a series of comparative tests will be used to determine the relative solubilities of the exhaust particles and both types of BeO. These studies will be composed of chemical analyses of all three materials, an *in vitro* solubility determination, and a single exposure nose-only inhalation study that will provide information on the deposition and clearance of all three materials.

### MATERIALS AND METHODS

The rocket motor exhaust materials (REM) were obtained from a rocket motor test facility. The material was scraped from the inlet section of an exhaust treatment facility designed to allow

the rocket motors to operate under conditions simulating actual operation while confining the exhaust materials to prevent atmospheric release. Material was collected from a series of test runs. The samples were packaged at the test site and sent to the THRU.

### Physical and Chemical Characterization

The REM was analyzed by spark source mass spectroscopy (SSMS) to obtain a profile of the chemical characteristics of material from each test. Beryllium content was determined by atomic absorption spectroscopy. Samples of the REM were examined by scanning electron microscopy, and the particle size was determined from the resulting micrographs. Geometric diameters of the particles were obtained using a Ladd Model 40000 Microprocessor Image Analyzer. The data obtained from measuring the apparent average diameter were converted to mass median aerodynamic diameter by assuming a density of 2.5 g/cc for the REM. If the particle size of the material was too large to be respirable (>3.0 µm mass median aerodynamic diameter), provisions were made to grind the material using an air-driven mill. The mill was chosen to avoid contamination or partial dissolution of the REM, which could result if a fluid grinding process was used. The ground material was used for the remainder of these studies as well as the *in vitro* studies discussed in Section 5.4 of this report.

Particle solubility will be determined by the method of Kanapilly et 'al. (1973). Specially designed flow-through filters will be loaded with REM and placed in a water bath at 37°C. A proteinaceous solution with solubility properties similar to those observed for serum will be allowed to flow past the filter sandwich. Table 5.3-1 compares this simulant with blood plasma. Samples of the serum simulant will be analyzed for Be as a function of time. From these data, a comparison of REM as well as low-fired and high-fired BeO solubility will be obtained.

Serum simulant and other Be-containing samples will be analyzed by chelating the Be ions with trifluoroacetylacetone (TFE) and subsequent gas chromatograph (GC) separation. The Be-containing TFE peaks will be quantified as Be per unit area. Insoluble samples will be digested in a Parr bomb prior to chelation.

### In Vivo Deposition and Clearance

For each material, a group of 40 rats will be exposed to the maximum attainable REM aerosol concentration using a nose-only exposure system. The REM will be aerosolized as a dry powder using a fluid-bed aerosol generator, unless solubility studies indicate that immersion of the particles in a fluid such as water will not alter their properties. If this is the case, the particles will be nebulized from a water slurry. Following exposure, the animals will be subjected to serial sacrifice on a schedule determined by the measured solubility of the REM. Urine and feces will be collected, from selected animals, to analyze their Be content. Since the endogenous levels of Be are low, the Be

found in these excretion products will reflect the clearance of Be from the respiratory tract. At sacrifice, selected tissues will be harvested and analyzed to directly determine their Be content. Comparison of these data will allow the relative bioavailability of Be to be determined from REM as well as high-fired and low-fired BeO.

### TABLE 5.3-1. COMPOSITION OF BLOOD PLASMA AND LUNG FLUID SIMULANT

| Chemical Species | Blood Plasma (mM/L) | Serum Simulant (mM/L) |
|---|---|---|
| Sodium | 142.0 | 145.0 |
| Potassium | 5.0 | -- |
| Calcium | 2.5 | 0.2 |
| Magnesium | 1.5 | - |
| Ammonium | - | 10.0 |
| Protein | 6.6 | - |
| Chloride | 103.0 | 126.0 |
| Bicarbonate | 27.0 | 27.0 |
| Phosphate | 1.2 | 1.2 |
| Citrate | 0.2 | 0.2 |
| Organic Acid | 6.0 | 6.0 |
| Sulfate | 0.5 | 0.5 |

| Chemical Compound | NaCl | NH$_4$Cl | NaHCO$_3$ | NaH$_2$PO$_4$ | Na$_3$Cit | Glycine | H$_2$SO$_4$ | CaCl$_2$ |
|---|---|---|---|---|---|---|---|---|
| mM/L | 116 | 10 | 27 | 1.2 | 0.2 | 6 | 0.5 | 0.2 |

### RESULTS

A total of ten sets of samples from the rocket test facility were received. Material was collected from several locations; however, the samples with the smallest starting particle size were collected from the regions of the test facility where the exhaust plume from the test engine struck the facility walls. For these studies, seven samples were selected, which were light gray in color, in contrast to the commercial BeO materials, which were white. A typical scanning electron micrograph of the REM is shown in Figure 5.3-1.

The mean geometric size of the particles was 144 μm (geometric standard deviation = 1.57), far too large to be respirable. Energy dispersive X-ray analysis was conducted while the samples were in the scanning electron microscope. This analysis method is not valid for light elements, but was able to identify phosphorus, chlorine, and sulfur present in the REM.
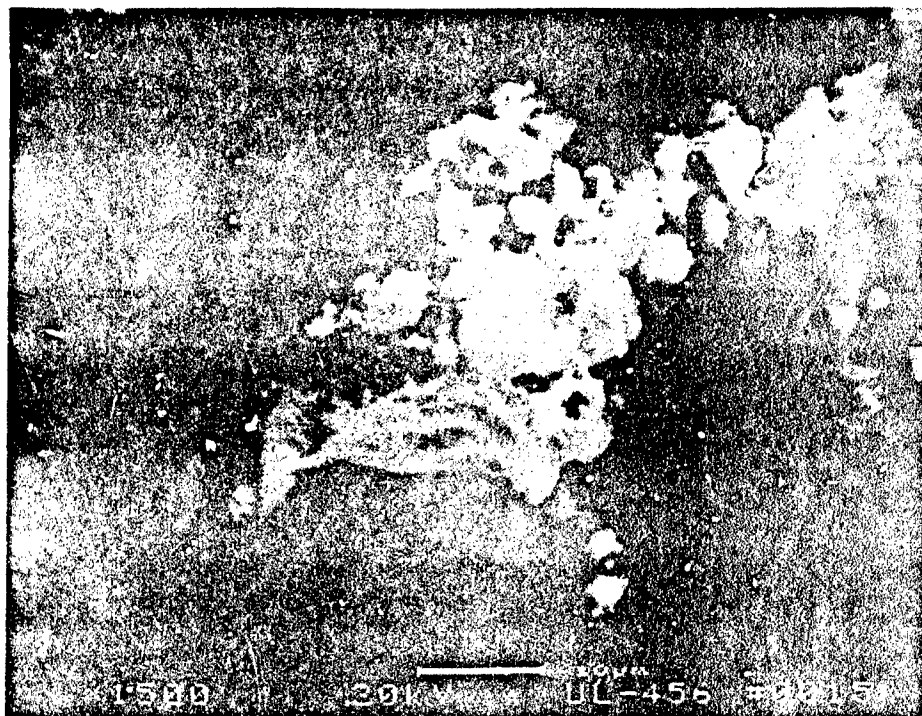
Figure 5.3-1. Scanning Electron Micrograph of Rocket Exhaust Particle.

These exhaust samples were also analyzed by SSMS. The materials, all found to be similar in composition, were pooled into one larger sample. Table 5.3-2 lists the major elements identified by SSMS. Concentrations of beryllium, aluminum, and magnesium were determined for the samples using atomic absorption spectroscopy. This analysis is presented in Table 5.3-3. Examination of the SSMS data in Table 5.3-2 indicates no clear differences in elemental composition among the samples that preclude combining them. Therefore, the REM samples were pooled to provide sufficient material for the remaining studies.

TABLE 5.3-2. ELEMENTAL COMPOSITION OF ROCKET MOTOR EXHAUST PARTICLES BY SOURCE MASS SPECTROSCOPY

| Sample # | B | Si | P | Cl | K | Fe | Mn |
|---|---|---|---|---|---|---|---|
| 1 | >4100[a] | 1100[a] | 290[a] | 210[a] | 190[a] | 3900[a] | 240[a] |
| 2 | >4100 | 280 | 1000 | 1100 | 230 | >1% | 2500 |
| 3 | >4100 | 100 | 820 | 1100 | 1100 | >1% | 98 |
| 4 | 2000 | 70 | 190 | 1400 | 1% | 3600 | 150 |
| 5 | 2000 | 100 | 1100 | 650 | 940 | 3600 | 49 |
| 6 | 1600 | 280 | 680 | 280 | 400 | >1% | 2800 |
| 7 | 2000 | 420 | 680 | 620 | 270 | >1% | 1300 |
| 8 | >4100 | 600 | 1400 | 280 | 230 | >1% | 2000 |
| 9 | >4100 | 600 | 680 | 740 | >1% | >1% | 4% |
| 10 | >4100 | 420 | 1400 | 2800 | 53 | >1% | 660 |

[a] parts per million unless otherwise indicated

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#### TABLE 5.3-3. ELEMENTAL COMPOSITION OF ROCKET MOTOR EXHAUST PARTICLES BY ATOMIC ABSORPTION

| Sample # | Be | | Al | Mg |
|---|---|---|---|---|
| 1 | 170,000[a] | (17%) | 130[a] | 330[a] |
| 2 | 170,000 | (17%) | 130 | 400 |
| 3 | 230,000 | (23%) | 120 | 270 |
| 4 | 200,000 | (20%) | 200 | 300 |
| 5 | 250,000 | (25%) | 140 | 220 |
| 6 | 68,000 | (6.8%) | 110 | 120 |
| 7 | 160,000 | (16%) | 110 | 340 |
| 8 | 160,000 | (16%) | 210 | 100 |
| 9 | 190,000 | (19%) | 140 | 860 |
| 10 | 220,000 | (22%) | 170 | 200 |

[a] parts per million

The manufacturer of the low and high temperature calcined BeO material supplied a chemical analysis of these materials, which is presented in Table 5.3-4.

#### TABLE 5.3-4. TRACE ELEMENT COMPOSITION OF LOW AND HIGH TEMPERATURE BERYLLIUM OXIDE

| Element | 550°C | 1600°C |
|---|---|---|
| B | 2 ppm | 1 ppm |
| Al | 40 ppm | 320 ppm |
| Cr | 2 ppm | 2 ppm |
| Fe | 10 ppm | 15 ppm |
| Mg | 5 ppm | 5 ppm |
| Mn | 2 ppm | 2 ppm |
| Ni | 3 ppm | 3 ppm |
| Ti | 4 ppm | 4 ppm |
| Na | 30 ppm | 60 ppm |
| Ag | 1 ppm | 1 ppm |
| Ca | 30 ppm | 30 ppm |
| Co | 1 ppm | 1 ppm |
| Cu | 2 ppm | 2 ppm |
| Mo | 3 ppm | 3 ppm |
| Pb | 2 ppm | 2 ppm |
| Si | 195 ppm | 1550 ppm |
| Zn | 20 ppm | 20 ppm |
| U | 30 ppm | 40 ppm |
| F | 1.46% | ND |

ND = not detected

## DISCUSSION

Since the as-received particle size was too large to allow an inhalation study to be conducted, the pooled material was ground using a Trost Jet Mill. This milling procedure reduced the particle size to ~3μm. A determination of actual particle size is currently underway.

Theoretical calculations predicted that a portion of the Be in the rocket motor would be converted to BeCl. Since BeCl is highly soluble and would contribute markedly to the dissolution of the REM, its presence in appreciable quantities would significantly increase the REM solubility relative to BeO. The amount of Cl found in the samples (~200 to 2800 ppm [Table 5.3-2]) was lower than predicted. The light gray color and the presence of high concentrations of B and Fe indicate that the REM particles also include impurities, probably generated by other combustion materials from the rocket fuel.

The material used in this study should provide an indication of the relative bioavailability of Be from REM when compared with BeO. However, these rocket exhaust particles may not be representative of the state of the particles that would be released during free flight of such a rocket. It can be anticipated that the collection of these materials on the vessel walls and their subsequent removal and grinding in the laboratory have altered the physical state of the particles. Nevertheless, the results of this study should provide guidance to the Air Force in determining the need for follow-up toxicological studies using materials more representative of the free flight rocket exhaust and in planning these studies.

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## 5.4 THE STUDY OF BERYLLIUM OXIDE GENOTOXICITY IN CULTURED RESPIRATORY EPITHELIAL CELLS

### V. Steele[a] and R. Kutzman

### INTRODUCTION

There are technical advantages in using beryllium (Be) solid propellant as fuel for rocket motors. However, beryllium oxide (BeO) in the exhaust of the rockets is a compound of primary toxicologic concern. An apparent difference between the toxicities of BeOs produced at low and high temperature firing has been reported, with the oxide produced at the higher temperature being the less toxic (Spencer, 1968). It also appears that the material produced in the exhaust of Be-fueled rockets is more like that produced at a high temperature.

The study is designed to provide information on the carcinogenic and genotoxic properties of rocket exhaust containing BeO particles. Beryllium oxide and $BeHPO_4$ have been shown to cause lung carcinomas and bone sarcomas by inhalation and by intravenous and intraosseous administration (IARC, 1980). This study, now in progress, uses rat tracheal epithelial (RTE) cells and measures the morphological transformation of these cells as an indicator of oncogenic transformation (Steele and Mass, 1985). Single stranded DNA breaks resulting from exposure of RTE cells to low and high temperature-fired BeO particles and rocket exhaust particles are also being assessed in the same cell type using the alkaline elution technique (Kohn et al., 1976). RTE cells are being used because tracheal epithelium would be expected to be a target tissue in exposed humans.

### MATERIALS AND METHODS

#### Test Material

The rocket exhaust residue sample (#4300-055-046-CS-3B) from Morton Thiokol, Inc., was provided by the United States Air Force (USAF) and is referred to as Be1 in this report. The low-fired BeO sample (#4300-055-048-LF-2) was produced by Brush Wellman, Inc., and is referred to as Be2 herein. The high-fired BeO (#4300-055-049-3) was also produced by Brush Wellman, Inc., and is referred to as Be3 herein. The test samples were milled to a small uniform size by the Toxic Hazards Research Unit at Wright-Patterson Air Force Base (WPAFB) and supplied to the Cellular and Molecular Toxicology Program at the Research Triangle Park (RTP) facility as fine powders in glass vials. These powders were weighed into sterile glass scintillation vials and suspended in Dulbecco's Modified Eagles Medium (DMEM) at 50 mg/mL. The glass vials were sterilized by autoclaving, and the

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final volume was measured. Sterile distilled water was used to replenish water lost during the sterilization process and to reestablish the 50 mg/mL stock concentrations of all three samples. All samples were insoluble in the culture medium, and extreme care was taken to ensure homogeneous mixing prior to sampling.

## Animals

Male Fischer 344 rats, between eight and twelve weeks old, were used in this study. Seven-week-old animals were supplied by Charles River Breeding Labs in Raleigh, NC, and were quarantined, for one week prior to initiation of the test, for health and disease evaluation. All evaluation tests were negative. All animals were housed in polycarbonate cages with hardwood Beta®-chip bedding. The animals were fed Purina® certified rodent chow #5002 and water *ad libitum*.

## Tracheal Cell Isolation

RTE cells were isolated by aseptically removing the trachea from young adult rats. The tracheas were cannulated with sterile polyethylene tubing and rinsed with Joklik's Modified Eagle Medium (JMEM). The lumen of each trachea was then filled with a protease solution and incubated overnight at 4°C. The following day the tracheal epithelial cells were rinsed from the tracheas with cold DMEM containing 10% fetal bovine serum (FBS). The cells were filtered to remove clumps, centrifuged, and resuspended in culture medium.

## Rat Tracheal Epithelial Focus Assay

To select concentrations for the RTE Focus Assay, a preliminary range-finding cytotoxicity assay was conducted. This assay measures reproductive survival (measured as colony-forming efficiency [CFE]) following exposure to a test agent. Approximately 20,000 cells were plated into collagen-coated 60-mm tissue culture dishes. The cells were cultured in a 1:1 mixture of Ham's F-12 medium and 3T3 conditioned DMEM + 2% FBS, containing insulin, hydrocortisone, transferrin, and bovine hypothalamus extract. At Day 1 the cultures were treated with 1000, 100, 10, 1, 0.1, 0.01, and 0.001 μg of BeO per milliliter of culture medium. $N$-methyl-$N'$-nitro-$N$-nitrosoguanidine (MNNG) dissolved in dimethyl sulfoxide (DMSO) was used as the positive control at a concentration of 0.6 μg MNNG/mL at 0.2% DMSO. Negative control cultures consisted of 0.2% DMSO alone and medium. Four dishes per group were used, and all three BeO test samples were tested on a single day with a single set of controls. Following a 24-h exposure, the medium was replaced with fresh medium. At Day 6 the cultures were fixed and stained with methylene blue. The colonies were counted with an Artec Automatic Colony Counter and the results recorded. The final data are expressed as percent of

control CFE. The concentration of test substance that reduced the colony count by 20% compared to control was designated as the highest concentration (HC) to be used in the assays.

The RTE focus assay follows a similar procedure except that the cultures will be treated with 30, 10, 3, 1, and 0.3 µg of BeO per milliliter of culture medium for 24 h at Day 1. The treatment with all three BeO samples will be conducted on the same day with concurrent controls. There will be 29 dishes in each of the 19 groups. Three dishes per group will be used for a parallel cytotoxicity assay. Six dishes per group will be used for the alkaline elution assay in the second week of the study. The remaining 20 dishes per group will be cultured until Day 30 and then fixed, stained, and scored for transformation frequency. A second independent duplicate assay also will be conducted. The transformation frequency will be calculated as follows,

$$Percent\,Transformation = \frac{Mean\,number\,of\,foci\,per\,dish \times 100}{Mean\,number\,of\,surviving\,colony-forming\,units}$$

where the number of colony-forming units per dish is determined by the coeval cytotoxicity assay.

### Rat Tracheal Epithelial DNA Damage Assay

An alkaline elution assay will be used to measure the ability of the three BeO samples to cause single strand DNA breaks in the RTE cells. The RTE cells, plated as described above, will be allowed to reach about 80% confluence. At that time the cultures will be treated for 24 h to the same concentrations as used in the focus assay, and positive control cultures will be treated with 1 mg cadmium sulfate/mL. Treated cells will then be harvested by trypsinization, resuspended, placed on a 0.2-µm polycarbonate filter, lysed, and eluted under alkaline conditions into ten 90-min fractions. The amount of DNA in each fraction will be quantified by a Hoechst dye technique using calf thymus DNA to generate a standard curve. DNA, which contains more single stranded breaks, will elute faster since it is smaller and passes more readily through the filter.

### Experimental Evaluation

The transformation frequencies of the test substances will be determined for each concentration by determining the number of transformed colonies per surviving cell. The maximum increased rate of DNA elution above the rate for control cells will be determined for each concentration of test material. The compounds will be considered positive for carcinogenicity if they (1) increase the mean transformation frequency to at least twice that of background levels and (2) increase the elution rate by 20% compared to controls. A second independent assay should confirm these results.

## RESULTS

The data from the range-finding cytotoxicity assay was evaluated and are presented in Figure 5.4-1. The media control had a CFE of 0.74 ± 0.02%, which represents an average of 148 colonies per 20,000 cells plated per dish. Typical dose-response curves were seen for all three samples, with the highest concentration (1000 μg/mL) resulting in greater than 98% toxicity. The actual rocket-exhaust test sample (Be1) and the low-fired BeO sample (Be2) were more toxic to RTE cells than the high-fired BeO sample (Be3). Based on these results, the greatest concentration chosen for the RTE Focus Assay was 30 μg/mL. At 30 μg/mL, the CFE of Be1- and Be2-treated cells was about 55%, and for Be3 treated cells it was about 80% that of control cells.

The RTE focus assay and the alkaline elution assays currently are being conducted, and the data are being analyzed.
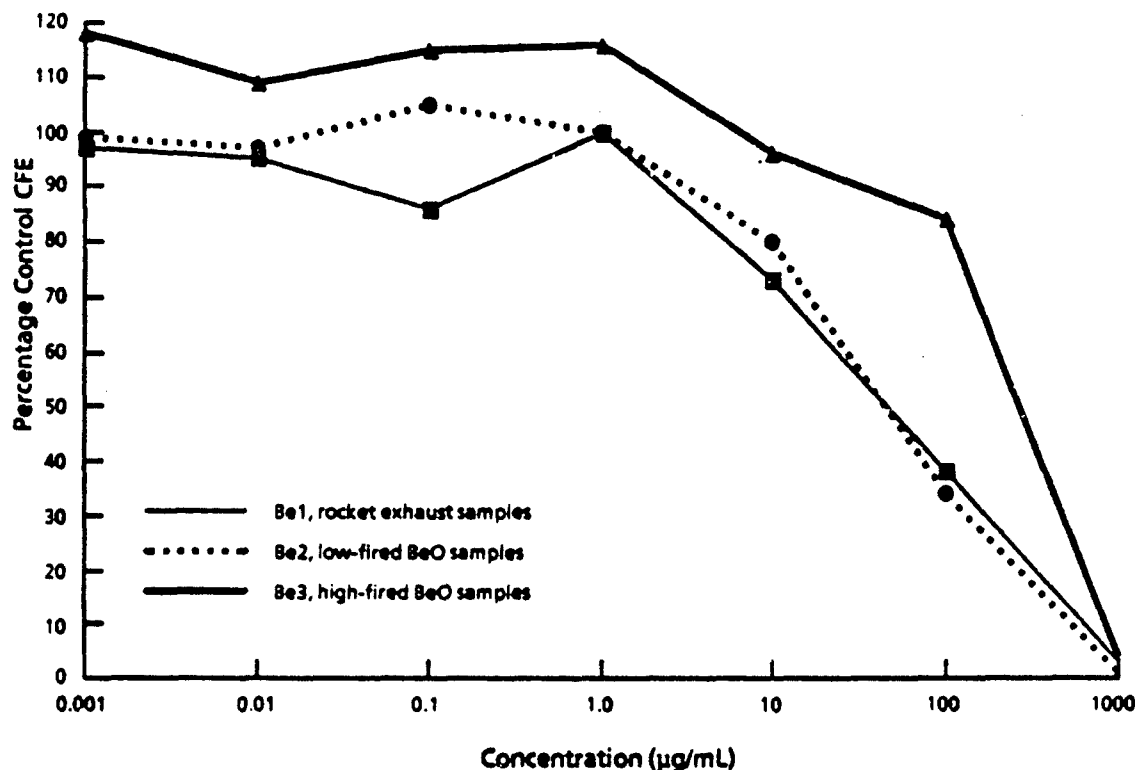


Figure 5.4-1. Cytotoxicity of Beryllium Oxide Samples Using Rat Tracheal Epithelial Cells. Control Colony-Forming Efficiency (CFE) was 0.74%.

## DISCUSSION

The results to date indicate that the BeO particles are toxic to respiratory epithelial cells at concentrations above 10 to 30 μg/mL. The rocket exhaust samples and the low-fired BeO samples

appear to be more toxic than the high-fired beryllium oxide particles. The data generated during these studies should permit comparison of the rocket exhaust particles to known BeO samples in regard to their cell transforming potential and ability to damage DNA.

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# SECTION 6

## PHARMACOKINETIC AND PHARMACODYNAMIC MODELING

### 6.1 COMPUTER SIMULATION OF CHEMICAL CARCINOGENESIS

R. Conolly, R. Reitz[a], H. Clewell, III[b], and M. Andersen[b]

*INTRODUCTION*

A number of chemicals used by the Air Force or found in groundwater at Air Force installations are rodent carcinogens. The methods used for estimating the human health risks associated with exposure to these chemicals are, for the most part, statistically based. These methods use little of what is known about carcinogen pharmacokinetics, biochemical mechanisms of action, and cancer biology. Nevertheless, they serve as a basis for estimating exposure standards, which are expected to protect the public health and may lead to enormous expenditures for compliance and remedial actions. The overall objective of the work described herein was to develop a computer simulation model, pharmacodynamic cancer model (PCM), for chemical carcinogenesis. Once validated with appropriate laboratory experiments, the PCM would incorporate scientific information on chemical carcinogenesis in a form that makes it available for use in risk assessment.

Computer simulation is a form of modeling in which the basic structure of a system is described mathematically within a computer program. These mathematical descriptions are usually complex and can not be solved exactly to describe the state of the system (i.e., its behavior) for any given point in time. Algorithms are available, however, which give approximate solutions to useful levels of precision. These solutions can be tested against experimental observations to ensure that the model is valid. Although simulation is widely used in the physical sciences to model processes ranging from airplanes in flight to the interactions of colliding galaxies (Hut and Sussman, 1987), it is infrequently used in the biological sciences.

The PCM includes information on chemical disposition in the organism, the nature of the interaction of the chemical with the target tissues, the dynamics of cell growth and death, and the uncontrolled growth of transformed malignant cells. Formulation of the model as a simulation program permits rapid examination of system behavior under a variety of conditions, some of which may not be accessible to direct experimental observation. With validation, the PCM could become a powerful tool for conducting mechanistically based carcinogen risk assessments.

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## PCM Structure

The overall PCM (Figure 6.1-1) consists of a sequence of three submodels

1. a physiologically-based pharmacokinetic submodel, which defines tissue dose by describing carcinogen absorption, distribution, and metabolism (Figure 6.1-1, Part I);

2. a two-stage cancer submodel in which cells are either normal, intermediate (1 mutation), or malignant (2 mutations). Tumor incidence is a direct function of the number of cells in the normal and intermediate populations, their birth and death rates and the transition frequencies between stages (Figure 6.1-1, Part III); and

3. biochemical mechanisms of action for DNA-damaging agents, promoters, and cytotoxic carcinogens. These mechanisms of action link the tissue dose of chemical carcinogen to the birth- and death-rate parameters and transitional probabilities of the two-stage cancer model (Figure 6.1-1, Part II).

## Pharmacokinetics of Chemical Carcinogens

The full simulation model has to track highly time-dependent changes in tissue concentrations of parent chemical and important metabolites. A number of investigators have shown that the pharmacokinetic behavior of drugs and toxic chemicals can be simulated accurately by what are commonly known as physiologically-based pharmacokinetic (PB-PK) models (Bischoff and Brown, 1966; Matthews and Dedrick, 1984; Ramsey and Andersen, 1964; Andersen et al., 1987). The essence of PB-PK models is the mathematical description of mammalian architecture and of the actual biochemical processes for metabolism and elimination. To the extent that this is done well, these models can not fail to produce accurate simulations of pharmacokinetic behavior. A PB-PK model, based on the one described by Ramsey and Andersen (1984), is part of the PCM (Figure 6.1-1, Part I). This PB-PK component simulates absorption, distribution, metabolism, and elimination to obtain target tissue concentrations of parent compound and metabolite(s).

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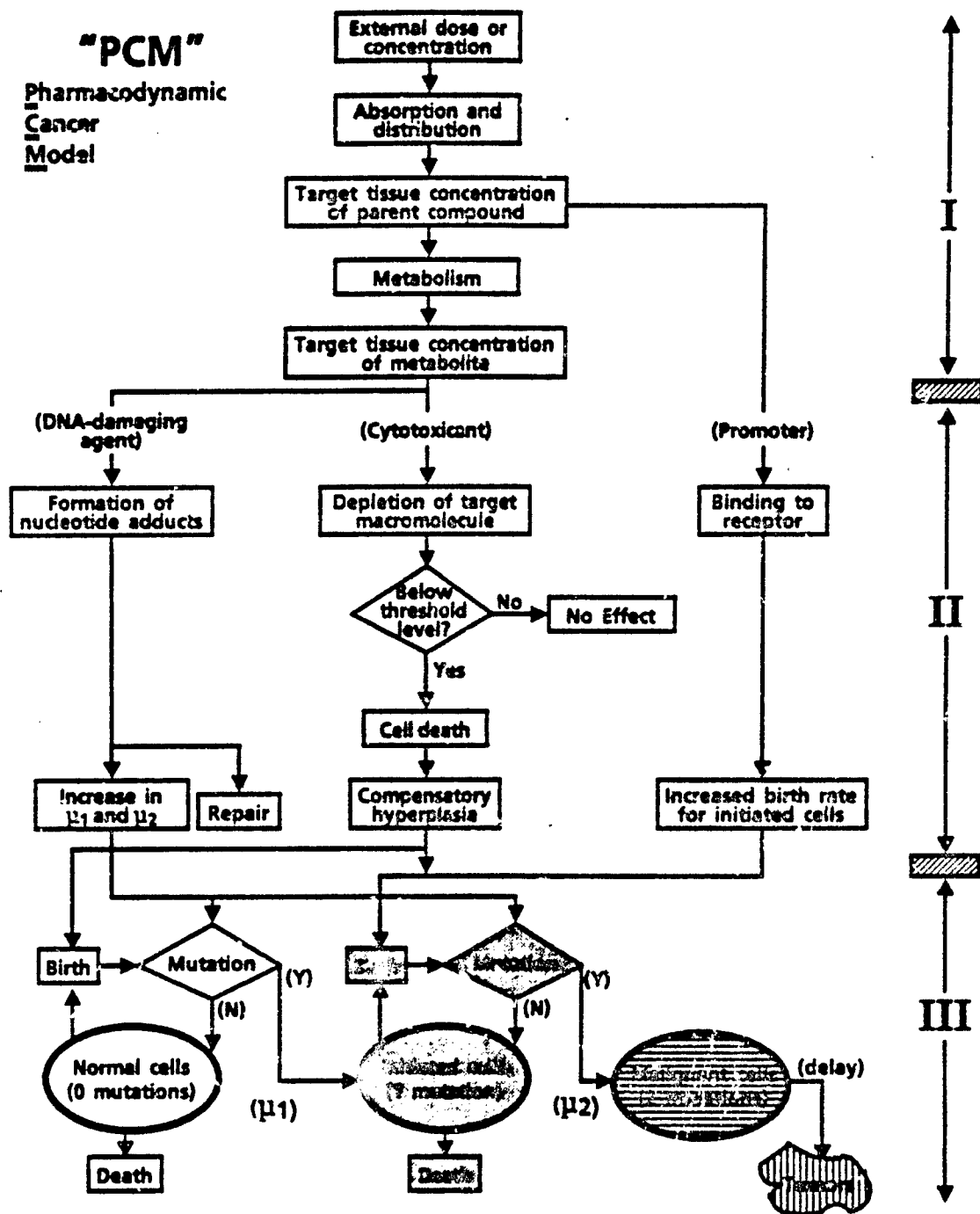
**Figure 6.1-1.** Schematic of the Pharmacodynamic Cancer Model (PCM). Part I is a physiologically based description of the processes controlling carcinogen pharmacokinetics. Its main role is converting external carcinogen dose to a realistic target tissue concentration of either the parent compound or relevant metabolites(s). In Part II, representative biochemical mechanisms are described for three distinct classes of carcinogens: DNA-damaging agents, promoters, and cytotoxicants. Part III consists of a two-stage cancer model where cells are either normal, intermediate, or malignant (0, 1, and 2 mutations, respectively). The linkages between Parts II and III specify how the different kinds of carcinogens affect the cell growth and mutation parameters of the cancer model.

## Numerical Two-Stage Cancer Model

A two-stage cancer model (M-K model) incorporating basic biological information on the cell growth characteristics of normal, initiated, and overtly malignant cells has been described (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981). This model can simulate the complex, age-specific incidence curves for breast cancer (Moolgavkar et al., 1980) and for childhood cancers such as retinoblastoma (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981). The model was used to identify different incidence behaviors expected for promoters rather than genotoxic chemical carcinogens (Moolgavkar, 1983). While the M-K model uses a combination of probabilistic and deterministic terms to approximate the tumor incidence curve, we have derived an entirely deterministic description. The crux of this description is a set of three continuous, differential equations describing changes in the amounts of normal ($N0$), initiated ($N1$), and malignant ($N2$) cells as a function of time.

$$\frac{dA_{N0}}{dt} = A_{N0}(\alpha_0 - \beta_0) \tag{1}$$

$$\frac{dA_{N1}}{dt} = A_{N1}(\alpha_1 - \beta_1) + \mu_1\alpha_0 A_{N0} \tag{2}$$

$$\frac{d(N1 \rightarrow N2)}{dt} = \mu_2\alpha_1 A_{N1} \tag{3}$$

In equations 1 and 2, birth rates for normal ($N0$) and initiated ($N1$) cells are determined by birth-rate constants ($\alpha_0$, $\alpha_1$) and the number of cells ($A_{N0}$, $A_{N1}$). Death rates are described analogously. In equation 2, the term $\mu_1\alpha_0 A_{N0}$ describes initiated cells arising through mutation of normal cells, and $\mu_1$ is the transitional probability for the $N0$-$N1$ transition. Solving these equations gives the numbers of cells at any point in time.

Equation 3 is different from equations 1 and 2 in that no attempt is made to describe the growth of malignant cells after they are formed. Rather, equation 3 only tracks the formation of malignant cells from initiated cells. The appearance of clinically evident tumors could be modeled using a time delay or a distribution of time delays after discrete $N2$ cells appear. For example, Moolgavkar et al. (1980) assumed that human female breast tumors become clinically evident five years after a malignant cell first arises. For the simulations presented here, we assumed a uniform delay such that the appearance of malignant cells is a useful surrogate for clinically evident tumors. This assumption is sufficient for illustrating qualitative aspects of model behavior. Modeling clinically evident tumors per se would be necessary, however, to obtain quantitatively accurate simulations with the PCM validated for a specific chemical.

$A_{N2}$ can be defined as an expectation for an average member of the population at risk (cohort). The Poisson distribution is used in the PCM to apportion this expectation to individual

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members of the cohort. By using this method, the probability ($P_{TUM}$) of individual members having one or more malignant cells (tumors) can be estimated. The estimate then can be used to obtain tumor prevalence ($P_{TUM} \times$ number in cohort). Tumor incidence is calculated as the change in prevalence over time. The validity of the numerical estimates of prevalence and incidence depends on the suitability of the Poisson distribution.

## Biochemical Mechanisms of Carcinogens

The pharmacokinetic and cancer submodels described above have been used as stand-alone models. This paper focuses on how to link these models to obtain the PCM, which was accomplished using biochemical mechanisms for a DNA-damaging agent, a promoter, and a cytotoxic carcinogen. In these mechanisms, the parent compound or its metabolite(s) interacts with specific cellular macromolecules, and these biochemical interactions, in turn, directly affect the cancer model parameters. These events are modeled as occurring in rat liver, which initially is composed only of normal hepatocytes. Then cells are described as dying and being replaced to maintain a stable population. The choice of liver as the target organ is only a matter of convenience; other tissues could be described similarly.

## Carcinogens That Form DNA Adducts

The biochemical mechanism modeled for a chemical that forms DNA adducts is outlined in Figure 6.1-1, Part II. The parent compound, a procarcinogen, is metabolized in the liver by an enzyme system having Michaelis-Menten kinetics. The metabolite is eliminated by a pseudo first-order process and also forms adducts with DNA nucleotides. The nucleotide adducts are repaired by a first-order process. For modeling simplicity, no special consideration is given to the spatial geometry of DNA relative to the site of carcinogen metabolism. Nucleotides are, in effect, treated as though they are in uniform solution throughout the cell. The following differential equation specifies the amount of nucleotide adducts ($A_{ANU}$) as a function of their time-dependent rates of formation ($k_{MNU}A_M A_{NU}$) and repair ($k_{RNU}A_{ANU}$).

$$\frac{dA_{ANU}}{dt} = k_{MNU}A_M A_{NU} - k_{RNU}A_{ANU} \tag{4}$$

The formation of adducts is described by a second-order rate constant ($k_{MNU}$), the amounts of metabolite ($A_M$) and of nucleotides ($A_{NU}$). The rate of adduct repair is given by the product of a first-order rate constant ($k_{RNU}$) and the amount of adducts ($A_{ANU}$). The solution to equation 4 is used to calculate the fraction ($F_{AD}$) of all nucleotides that have adducts. This fraction is then multiplied by a term ($\mu_{MAX}$) defining the maximum possible increase in transition probabilities due to the DNA-damaging agent. In effect, $\mu_{MAX}$ reflects the mutational potency of the adducts. The result ($F_{AD}\mu_{MAX}$) is added to the transition probabilities $\mu_1$ and $\mu_2$ (equations 2 and 3). However, the birth- and death-rate parameters $\alpha_0$, $\beta_0$, $\alpha_1$, and $\beta_1$ are not affected. Consequently, normal-initiated cell and initiated-malignant cell transitions are more likely during and for some time after

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genotoxicant exposure. This mechanism of action is well-supported by the literature (McCann, et al., 1975; Barrett et al., 1978; Weinstein, 1981; Reddy, 1983). Elaborations of this formulation could describe the formation of more than one adduct per nucleotide and changes in transition probabilities corresponding to such multi-adduct nucleotides. Adducts on the sugar-phosphate backbone of DNA also could be considered. Although such elaborations may be appropriate for specific chemicals, they are not the main focus of this investigation, which is designed to illustrate how these processes can be described mathematically and integrated into a working model for chemical carcinogens.

### Promoters

The biochemical mechanism modeled for promoters is shown in Figure 6.1-1, Part II. Promoters are described as agents that increase tumor incidence by conferring a growth advantage on intermediate (N1) cells. This effect is achieved in the PCM by increasing the birth rate of N1 cells while leaving the death rate unchanged. Equation 5 describes the essential features of this mechanism:

$$\frac{P_{KBN1}}{dt} = F_{PRE}K_{BMAX} \qquad (5)$$

The promoter binds reversibly to a receptor, which is distributed throughout the cell. $P_{KBN1}$, the maximum possible increase in N1 birth rate, is defined as the fractional occupancy of the receptor ($F_{PRE}$) multiplied by the term $K_{BMAX}$. In this scheme, promoters have no effect on $\mu_1$ and $\mu_2$ nor on the growth characteristics of normal cells. This mechanism of action for promoters is consistent with the experimental observation that cells in preneoplastic foci (intermediate cells in the PCM) grow faster than normal cells (Schulte-Hermann et al., 1983) and with postulated biochemical mechanisms for 2,3,7,8-tetrachlorodibenzo-p-dioxin (Gasiewicz and Rucci, 1984) and phorbol esters (Weinstein, 1981).

### Carcinogens That Are Primarily Cytotoxic

For cytotoxicants, binding a parent compound metabolite to a target macromolecule (T) is described as the primary cytotoxic event. Cell viability depends on T and diminishes as normal T is progressively depleted. Cell death occurs only after many "hits" have occurred (i.e., after T has been depleted below some threshold level, which is less than the normal level of T in the cell). This general mechanism of cytotoxicity has been suggested for numerous compounds (Pohl, 1979; Boyd, 1980; Hinson, 1980; Jollow et al., 1974). The threshold level of T is modeled to vary among individual cells according to the normal distribution. The choice of the normal distribution is consistent mathematically with the commonly observed log-normal relationship between dose and toxic effect (Andersen, 1987). However, other relationships between the concentration of T and the probability of cell death (e.g., monotonically increasing) could be used equally well for heuristic purposes.

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When validation data for the PCM are obtained, the mathematical description of the normal distribution will be replaced by a "table function," which will be derived from the actual data by an algorithm supplied with the simulation language.

Several equations used to describe cytotoxicity are given below.

$$\frac{dC_T}{dt} = K_{ST} - K_{LT}C_T - K_{MT}C_MC_T \tag{6}$$

$$K_{KILL} = \int_{CT_0}^{CT} N(C_{TM}, \sigma^2 T)dT \tag{7}$$

$$A_{CSC} = A_{CO}K_{KILL} - T_X \tag{8}$$

$$\frac{dN0}{dt} = A_{N0}(\alpha_0 - \beta_0) + F_{N0}(K_{BTX}T_X - A_{CSC}K_{DU}) \tag{9}$$

$$\frac{dN1}{dt} = A_{N1}(\alpha_1 - \beta_1) + F_{N1}(K_{BTX}T_X - A_{CSC}K_{DU})$$

$$+ \mu_1(\alpha_0 A_{N0} + F_{N0}K_{RTX}T_X) \tag{10}$$

$$\frac{d(N1 \rightarrow N2)}{dt} = \mu_2(\alpha_1 A_{N1} + F_{N1}K_{BTX}T_X) \tag{11}$$

The concentration of $T$ ($CT$) is described by equation 6. $T$ is produced by zero-order synthesis ($K_{ST}$) and lost either through a first-order pathway ($K_{LT}C_T$) or through attack by a carcinogen metabolite ($K_{MT}C_MC_T$), where $K_{MT}$ is a second-order rate constant and $C_M$ is the concentration of metabolite.

Equation 7 describes the fraction of target tissue cells ($K_{KILL}$) in which $T$ has been depleted below the threshold level. To calculate $K_{KILL}$, a normal distribution is first defined where $C_{TM}$ is the concentration of $T$ as the mid-point of the normal distribution and $\sigma^2 T$ is the variance in units of $T$. $K_{KILL}$ is calculated as the area under the curve from $CT_0$ to the concentration at $T$ ($C_T$). As configured for most of the simulations presented here, the concentration of $T$ at time $= 0$ ($CT_0$) was set at 1000 µM, $C_{TM}$ at 650 µM, and $\sigma^2 T$ at 50 µM. These values were set arbitrarily and will be adjusted appropriately as data for specific compounds become available. An approximation given by Hastings (1955) is used to solve the integral. We recognize that validation studies are unlikely to identify a single macromolecule whose depletion leads to cell death. It seems more probable that a correlation will be found between binding to a class of macromolecules, perhaps proteins in a

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cellular subfraction, and cell death. In any event, validation studies can be reasonably expected to show a correlation between covalent binding and cell death, which could be used for deriving a table function.

Equation 8 describes the number of cells in the target tissue ($A_{CSC}$) where the concentration of $T$ is below threshold and which are therefore "available" to die. $A_{CSC}$ equals the basal number of cells in the tissue ($A_{C0}$) times $K_{KILL}$ minus the number of cells ($T_X$) that are "missing" from the tissue due to cytotoxicity. This difference can, of course, have a negative value. When it does, $A_{CSC}$ is forced to 0.

Equations 9, 10, and 11 are merely equations 1, 2, and 3 modified to account for toxicity-related changes in the birth and death rates of normal, initiated, and malignant cells. In equations 9 and 10, the rate of cell killing due to cytotoxicity is given by the product of the first-order terms $K_{DU}$ and $A_{CSC}$. These terms are multiplied further by either $F_{N0}$ or $F_{N1}$, which represent the fractions of the whole liver made up of normal and intermediate cells, respectively. The model is structured so that $F_{N0}$ and $F_{N1}$ always sum to 1.0. Malignant cells are modeled not to be susceptible to cytotoxicity. Regenerative hyperplasia is described by a first-order term ($K_{BTX}$) multiplied by $T_X$ and $F_{N0}$ and $F_{N1}$. In equations 10 and 11, the terms containing $\mu$ generate the cells appearing through mutations, which arise during regenerative hyperplasia. Similar approaches to modeling cytotoxicity have been described previously by Conolly et al. (1987) and Reitz et al. (1988).

### Species-Specific Behavior

The PCM embodies a generic description of mammalian architecture. To simulate experimental work with a particular species, physiological and biochemical parameter values appropriate for that species must be used. This approach has enabled physiologically-based disposition models, like the one incorporated in the PCM, to be enormously successful in predicting pharmacokinetic behavior of toxic chemicals across different mammalian species (Ramsey and Andersen, 1984; Andersen et al., 1987; King et al., 1986; Dedrick, 1973; Bischoff et al., 1971). Some PCM parameters, such as cardiac output and the sizes of major organs, are well known and also scale between species according to well-defined allometric relationships. Other parameters, for example, birth and death rates of initiated cells, are not known precisely. While allometry is often useful, it is preferable to set species-specific parameter values using data obtained with the target species. This practice precludes the possibility that unnoticed failure of an allometric relationship might invalidate model behavior.

For the simulation described here, the PCM parameters were scaled for a 250-g rat. Physiological parameters controlling carcinogen disposition throughout the organism were set to realistic values as described by Ramsey and Andersen (1984). The liver was modeled as the target organ for carcinogens, and specific hepatic parameter values were obtained whenever possible. Hepatocyte volume was set at 5.608E-12 L/cell (de la Iglesia, et al., 1975), and the total number of

hepatocytes/liver was calculated based on liver equaling 4% of body weight. This calculation was used to establish the number of normal cells at the start of each simulation. Hepatocyte birth and death rates were set at 1.74E-4 events/h/cell based on microautoradiography data collected by Reitz (1987). The number of nucleotides per hepatocyte was calculated based on 2.9E9 base pairs per human cell as quoted by Darnell et al. (1986). The basal transition probabilities $\mu_1$ and $\mu_2$ were set at 1.0E-6 per cell generation (Elmore et al., 1983; Tsutsui et al., 1981). Other parameter values for which data were not available were estimated. For example, the concentration of receptor to which a promoter binds was set at 1.0 μM.

At the present stage of development, when the PCM has not been validated for specific chemicals, the actual values set for many of the parameters are not critical. The qualitative behavior of the model is not particularly sensitive to fairly wide variations in many parameter values. This makes it possible to evaluate model performance before validation for specific chemicals is complete. Of course, quantitative aspects of the PCM simulations are dependent entirely on specific parameter values. The accuracy of simulated tumor incidence data for specific chemicals will always reflect the quality of the data used to set parameter value.

For the simulations described below, the PCM was configured to simulate inhalation exposure to a chemical having the pharmacokinetic behavior of chloroform. Tissue:blood and blood:air partition coefficients and metabolism rate constants for chloroform in male Fischer 344 rats were used (Gargas, 1987). The pharmacokinetic portion of the PCM (Figure 6.1-1, Part I) has been well validated for these types of exposures (Ramsey and Andersen, 1984; Andersen et al., 1987), so the tissue levels of chloroform achieved during these exposures were, therefore, realistic. It is not appropriate, however, to think of these simulations as reflecting expected consequences of chloroform exposure. No data are yet available to validate the cytotoxicity component of the PCM of chloroform. Furthermore, there is no evidence in the literature to suggest that chloroform acts by either of the DNA-damaging or promotional mechanisms in the PCM. For simulated experiments with a DNA-damaging agent and with a promoter, appropriate parameter values were set so that the test chemical had the capacity either to damage DNA or to act as a promoter. The test chemical used for these simulations should, therefore, be thought of as no more than a generic probe of model behavior.

Parameter values were always set so that the test chemical only worked by one mechanism of action at a time. It is probable that specific chemicals do have multiple mechanisms of actions. For example, diethylnitrosamine is both an acute cytotoxicant and a potent DNA-damaging agent (Richardson and Swenberg, 1987). It would not be difficult to configure the PCM for such chemicals, but that is beyond the scope of this report.

*Biochemical Interactions*

The PCM can be used to examine time-dependent macromolecular interactions, which comprise the biochemical mechanisms of carcinogens. The ease with which simulation models can be exercised and parameter values changed means that it is relatively simple to evaluate behavior of a given mechanism over a range of conditions. The basic description of a mechanism can also be changed readily. This allows the implications of different mechanisms for tumor incidence and biochemical parameters to be examined. An exhaustive treatment of the mechanisms described for DNA-damaging agents, promoters, and cytotoxicant carcinogens is not possible here, but several examples are provided to illustrate some possibilities.

Figure 6.1-2 shows aspects of the biochemical mechanism modeled for DNA-damaging agents. The relationship between inhalation exposure concentration, arterial blood concentration ($C_A$), and concentration of DNA adducts ($C_{ANU}$) is illustrated when all model parameter values are held constant except for the rate of DNA repair ($K_{RNU}$). The simulated exposure regimen was six h/day, five days/week for two weeks. The daily rise and fall in $C_A$ is clearly seen as well as the corresponding fluctuation in $C_{ANU}$. When $K_{RNU}$ was set to 0.01 h-1 (upper curve), DNA adducts accumulated during the week and were not completely repaired during the weekend. With $K_{RNU}$ set at 0.05 h-1 (lower curve), adduct accumulation during the week was diminished and was repaired almost totally on the weekend.
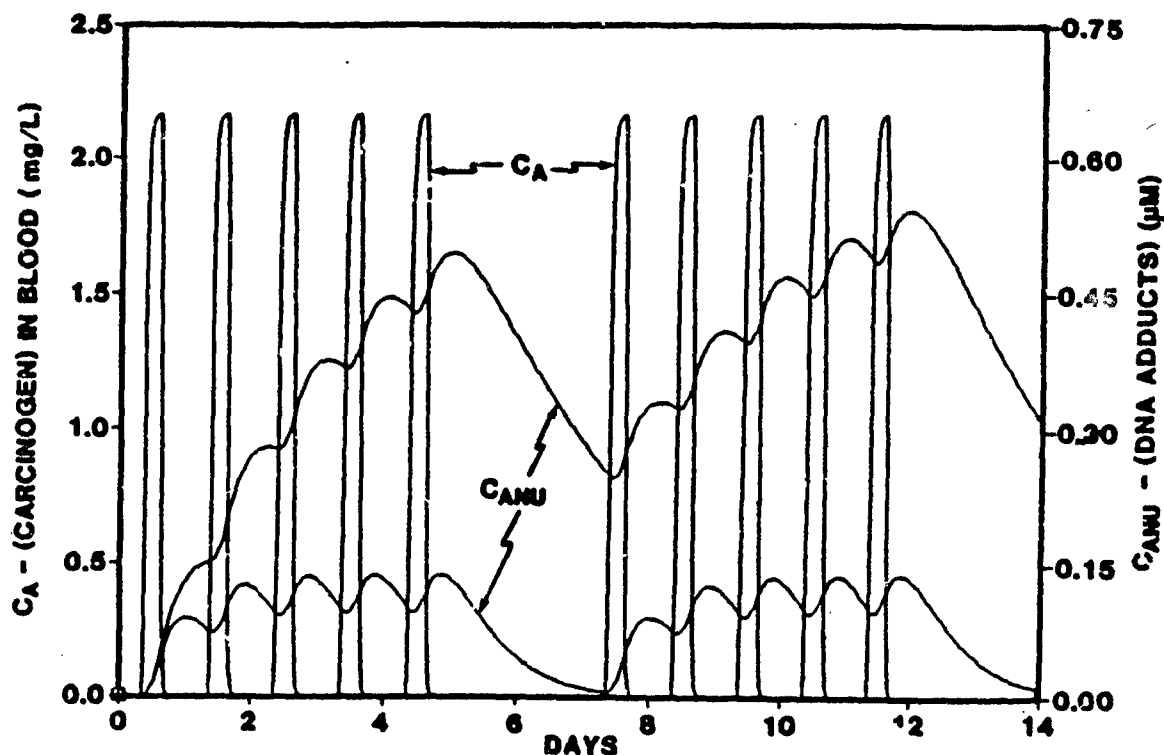


Figure 6.1-2    Kinetics and DNA-binding behaviors for DNA-damaging agent. The relationship between the arterial concentration of carcinogen ($C_A$) and the concentration of DNA adducts ($C_{ANU}$) is illustrated when all model parameter values are held constant except the rate of DNA repair. The simulated exposure regimen was 6 h/day, 5 days/week for 2 weeks. The rate of repair was 0.01/h (upper curve) or 0.05/h.

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Simulation of the interaction of a promoter with its receptor and the consequence of this interaction for the growth of initiated cells is shown in Figure 6.1-3. The exposure regimen was 2 years at 6 h/day, 5 days/week. The interval from day 700 through day 712 was chosen for display because the effects on initiated cells are illustrated more clearly once a significant population of these cells has developed. In the figure, daily spikes in the fraction of receptor molecules occupied by promoter ($F_{PREL}$) mirror the daily 6-h exposure to promoter. Each spike of receptor occupancy is accompanied by a sharp increase in the number of initiated cells ($A_{N1L}$), reflecting their temporarily increased birth rate.
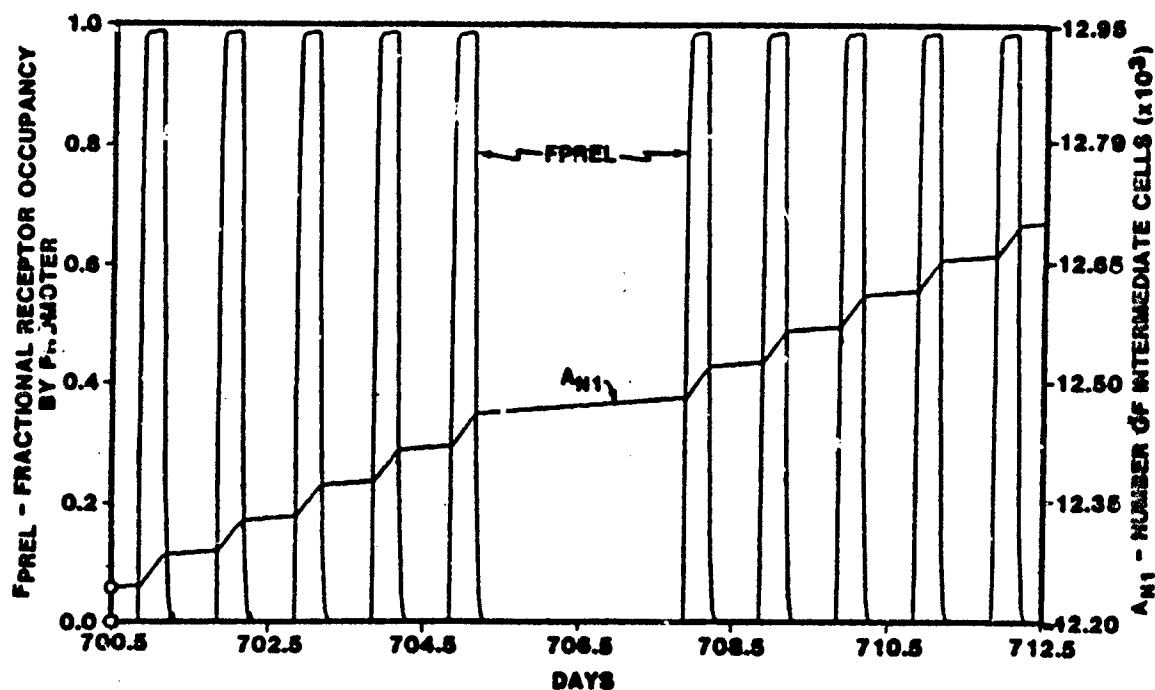


Figure 6.1-3    Interaction of a promoter with its receptor and the growth of initiated cells ($A_{N1}$). The exposure regimen was 2 years at 6 h/day, 5 days/week. Daily spikes in the fraction of receptor molecules occupied by promoter ($F_{PREL}$) mirror the daily 6-h exposure to promoter. Each spike of receptor occupancy is accompanied by a sharp increase in the number of initiated cells.

Effects of cytotoxicant on the target macromolecule (T) and on the number of normal cells in the liver ($A_{N0}$) are shown in Figure 6.1-4. Two weeks of exposure at six h/day, five days/week were simulated. The daily spikes in arterial concentration of cytotoxicant ($C_A$) are followed by a corresponding decline in the level of T. T is destroyed by a metabolite of the cytotoxicant. Resynthesis of T is not complete within 24 h, but full recovery does occur over the weekend. A similar pattern is seen for AN0, illustrating how viability of normal cells depends on the concentration of T.
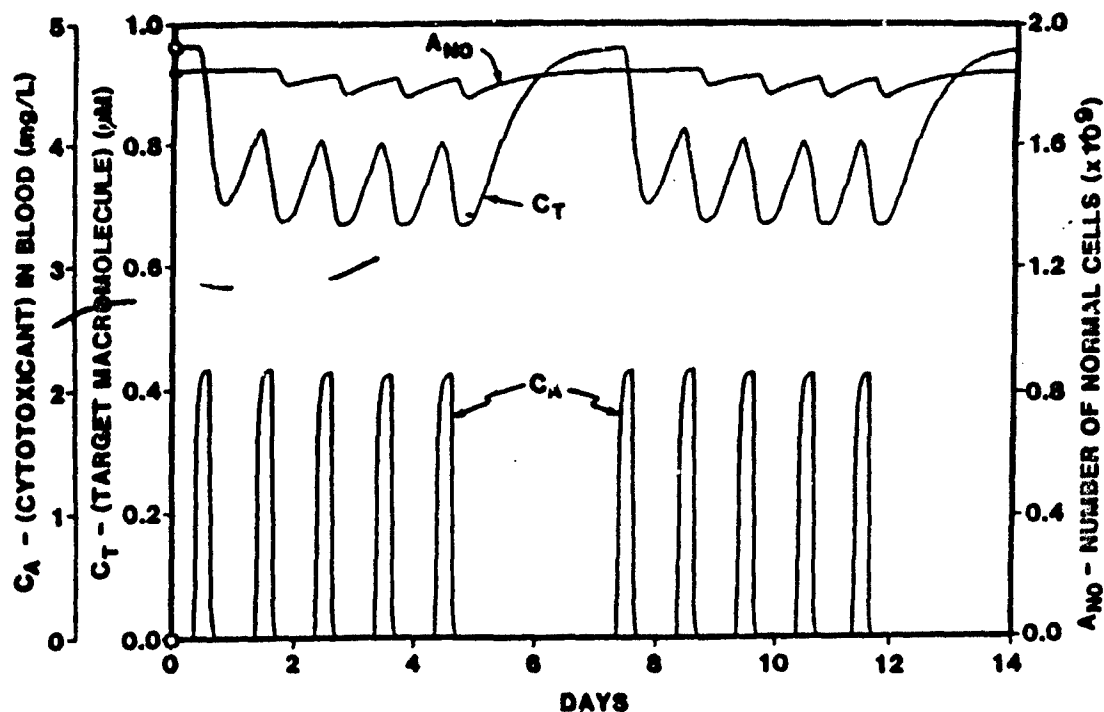
Figure 6.1-4    Arterial concentration of cytotoxic carcinogen ($C_A$), cellular concentration of target
macromolecule ($C_T$), and number of normal cells ($A_{NO}$). Two weeks of exposure at
6 h/day, 5 days/week were simulated. Cytotoxicant metabolism generates a reactive
metabolite that depletes the target macromolecule. Death of normal cells is
followed by regenerative hyperplasia.

## Tumor Prevalence and Dose-Response Curves

In Figure 6.1-5 tumor prevalence curves are plotted for a DNA-damaging agent, a promoter,
and a cytotoxicant. The exposure regimen was 6 h/day, 5 days/week for 2 years. Parameter values
were set to provide a tumor prevalence close to 100% for each agent at two years, which allows a
comparison of the shapes of the curves. Curve A shows the background prevalence, which totaled
about 0.8%. The model was configured so that background prevalence was due solely to the regular
turnover of normal and initiated cells. Curve B shows tumor prevalence for a promoter, whereas
curves C and D are for cytotoxicants and DNA-damaging agents, respectively. The shapes of the
curves for cytotoxicants and DNA-damaging agents are similar, as would be expected given the
interactions of these classes of agents with parameters controlling cell dynamics (Figure 6.1-1,
Part III). With the promoter, tumor prevalence rises more slowly in the early months of the
simulated experiment. The shape of the prevalence curve for the promoter reflects the fact that, as
modeled in the PCM (Figure 6.1-1, Parts II and III), a promoter will have a minimal effect on
prevalence until a relatively large population of initiated cells has developed.

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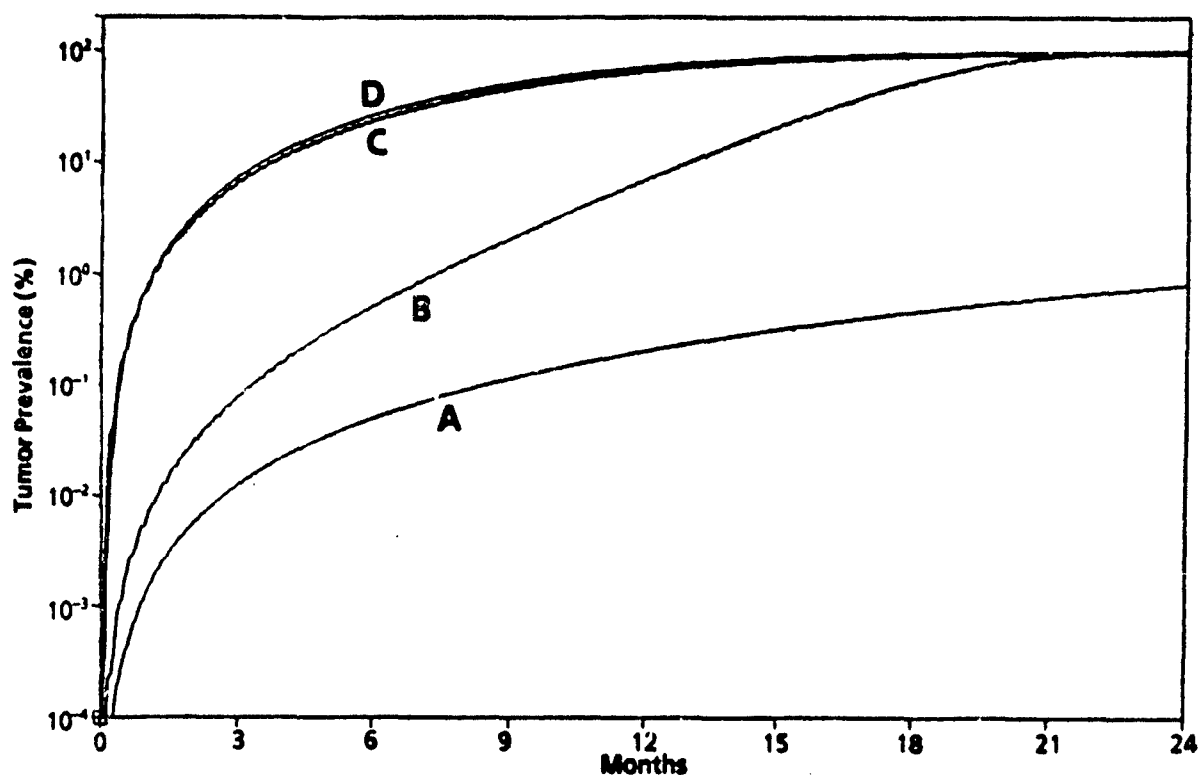
Figure 6.1-5    Tumor prevalence curves for a DNA-damaging agent, a promoter, and a cytotoxicant. The exposure regimen was 6 h/day, 5 days/week for 2 years. Prevalence elicited by each agent at 2 years was close to 100%. This allows a comparison of the shapes of the curves. Curve A is background prevalence (about 0.8%). Curves B, C, and D are for a promoter, cytotoxicant, and DNA-damaging agent, respectively.

Dose-response curves are illustrated in Figures 6.1-6. Each data point on these curve represents tumor prevalence at the end of a two-year bioassay. For these simulations, continuous exposure was modeled in order to save computer time (simulations run much faster when abrupt changes in parameter values, as occurs with intermittent exposure, are avoided). Switching between intermittent and continuous exposure affects the magnitude but not the shape of prevalence and dose-response curves. To obtain low-dose tumor prevalence due to carcinogen exposure, it was necessary to subtract the background prevalence from each data point. In Figure 6.1-6a, for example, background prevalence at 1.0E-3 ppm of the DNA-damaging agent was about three orders of magnitude higher ($\approx 0.8\%$) than the incidence due to the carcinogen ($\approx 0.0005\%$).

Figure 6.1-6a shows dose-response curves for a DNA-damaging agent. For curves A, B, and C, both basal transition probabilities ($\mu_1$ and $\mu_2$) were 1.0E-6 (mutations/division). Maximum possible increases in transition probabilities due to the carcinogen were 0.1, 0.1, and 1.0 (mutations/division), for curves A, B, and C, respectively. (This maximum possible increase can be thought of as a measure of mutational potency.) The actual increase in transition probability due to carcinogen was obtained by taking the product of maximum possible increase with the fraction of all nucleotides having adducts. The first-order rates of DNA repair were 1.0, 0.1, and 0.1 (1/h) for curves A, B, and C,

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respectively. As would be expected, increasing mutational potency increased tumor prevalence, whereas increasing the rate of DNA repair had the opposite effect. The linearity of these dose-response curves on log-log plots reflects the absence of a threshold in the mechanism modeled for DNA-damaging agents.

Dose-response curves for promoters are shown in Figure 6.1-6b. For curves A, B, and C, the basal birthrate for intermediate cells was 1.7E-4 (1/h). The maximum possible increases in intermediate cell birthrate due to the promoter were 1.7E-4, 1.7E-4, and 1.7E-2 (1/h) for curves A, B, and C, respectively. (This maximum possible increase can be thought of as a measure of the potency of the promoter.) The dissociation constants for the promoter-receptor complex were 10.0, 0.1, and 0.1 ($\mu$M) for curves A, B, and C, respectively. The actual increases in birthrate were obtained by taking the product of the maximum possible increase in birthrate and the fractional occupancy of the receptor. It can be seen that decreasing the affinity of the promoter for the receptor decreases tumor prevalence, whereas increasing promoter potency increases prevalence. There is no suggestion of a threshold for these curves. This result is, of course, a consequence of the mechansim modeled in the PCM. Promotional mechanisms where some minimum occupancy of the receptor is required before any effect occurs would show a threshold and could be readily incorporated into the model.

Figure 6.1-6c shows a family of dose-response curves for a cytotoxicant. These curves were generated by changing the standard deviation of the normal distribution ($\sigma_T$; equation 8). For curves A through D, the standard deviation was set at 80, 100, 120, and 140 $\mu$M target macromolecule, respectively. The midpoint of the normal curve was 550 $\mu$M. These parameters together determine the number of cells that will die from cytotoxicity at a particular concentration of macromolecule. For example, for the simulations shown in Figure 6.1-6e, the basal number of cells in the liver was 1.85E9, and the basal concentration of target macromolecule was 963 $\mu$M. At 16-ppm inhalation exposure concentration, the concentration of target macromolecule was 888 $\mu$M and the number of cells/liver below threshold was 1102; 93,177; 1.104E6; and 5.091E6 for curves A through D, respectively.

There has been a long debate over the shape of carcinogen dose-response curves in the low dose region where experimental investigation is not practical. It has been suggested, for example, that the risk associated with exposure to carcinogens that must undergo metabolic activation should not be calculated using the same extrapolation procedures that are used for directly-acting carcinogens (Reitz et al., 1982; Reitz et al., 1978). Figure 6.1-6 shows how the PCM can be used to explore this issue. Dose-tumor prevalence curves for different biochemical mechanisms are simulated over a range of exposure concentrations extending from those commonly used for rodent carcinogen bioassays down to the parts-per-billion concentrations often found at sites of environmental pollution.
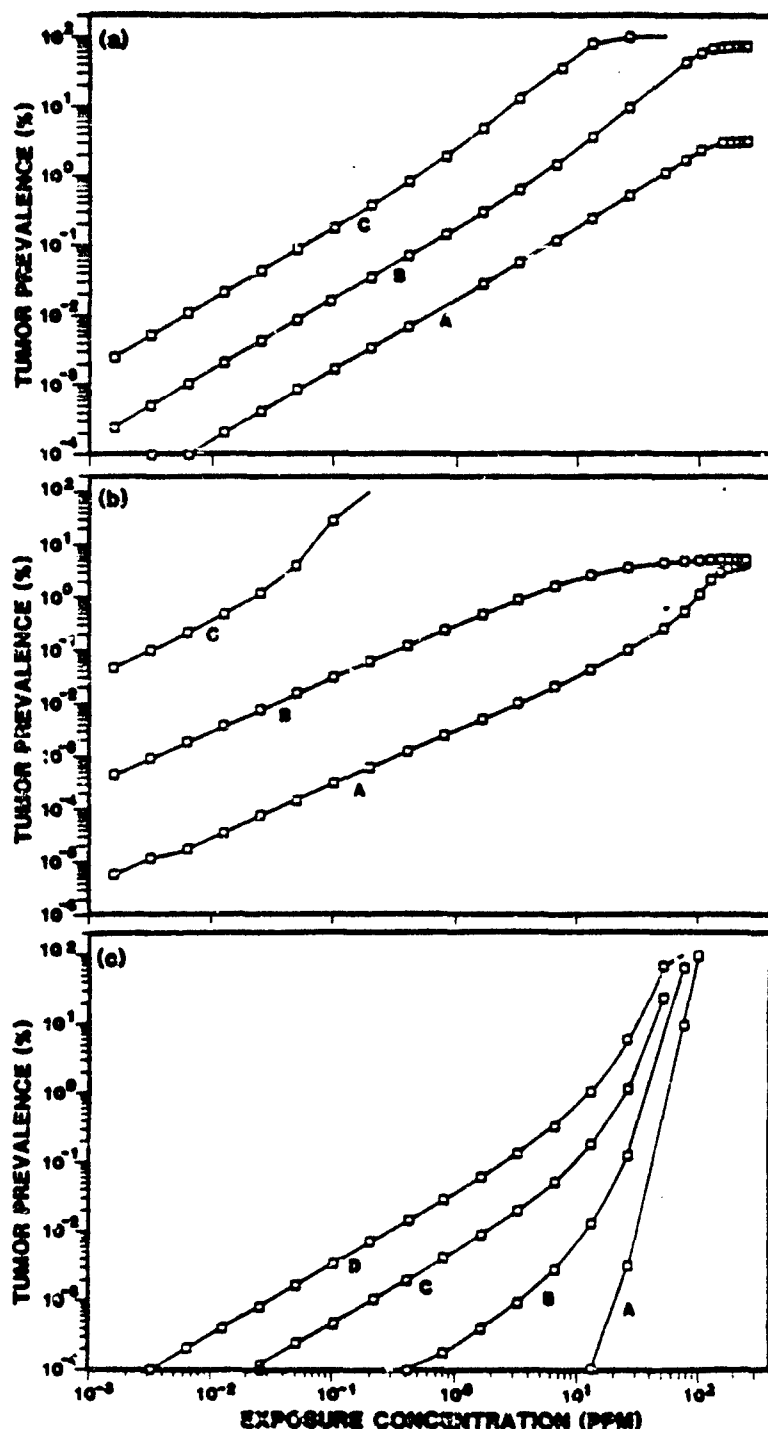
**Figure 6.1-6**
Dose-response curves for a DNA-damaging agent (a), a promoter (b), and a cytotoxicant (c). These curves represent tumor prevalence at the end of a simulated 2-year bioassay over a range of exposure concentrations. To see low-dose tumor prevalence due to the carcinogen, background prevalence ($\approx 0.8\%$) was subtracted from the calculated curves. DNA DAMAGE (6a): The maximum possible increases in transition probabilities due to carcinogen exposure were 0.1, 0.1 and 1.0 (mutations/division), for curves A, B, and C, respectively. The corresponding, first-order rates of DNA repair were 1.0, 0.1, and 0.1 (1/h). PROMOTION (6b): Maximum possible increases in intermediate cell birth rate due to the promoter were $1.7 \times 10^{-4}$, $1.7 \times 10^{-4}$, and $1.7 \times 10^{-2}$ (divisions/cell/h) for curves A, B, and C, respectively. The corresponding dissociation constants for the promoter-receptor complex were 10.0, 0.1, and 0.1 ($\mu$M). CYTOTOXICITY (6c): These dose-response curves were generated by changing the standard deviation of the normal distribution ($\sigma_T$). For curves A-D, the standard deviation was set at 80, 100, 120, and 140 $\mu$M target macromolecule, respectively. The midpoint of the normal distribution was 550 $\mu$M.

(a)

TUMOR PREVALENCE (%)

C

B

A

(b)

TUMOR PREVALENCE (%)

C

B

A

(c)

TUMOR PREVALENCE (%)

D

C

B

A

EXPOSURE CONCENTRATION (PPM)

## PCM Validation

The PCM is structured so that, in principle, all model parameters can be measured directly in the laboratory. In fact, much of the structure of the PCM, which was incorporated from pre-existing models, is already validated for those models (Ramsey and Andersen, 1984; Andersen et al., 1987; Moolgavkar and Knudson, 1981; Moolgavkar et al., 1980). The major segment of the PCM that is not validated at this time is that describing the biochemical mechanisms of action for DNA-damaging agents, promoters, and cytotoxicants. Our laboratories are currently attempting to validate the PCM for chloroform using the working hypothesis that chloroform carcinogenesis is secondary to repeated cytotoxic insults. Experimental programs could also be designed to validate the model with respect to promoters and genotoxicants.

We do not underestimate the challenges of the laboratory studies needed to validate the PCM. Necessary experiments include quantitating rates of DNA adduct formation and repair, identifying initiated cells, quantitating initiated cell birth and death rates, and defining the relationship between the first appearance of a malignant cell and of a clinically observable tumor. These experiments, however, are feasible with currently available technology.

An important aspect of simulation models with a biologically based structure is that they can assist in their own validation. For example, simulated experiments can be run using a range of initial conditions to determine which design is likely to provide useful output. This facet of simulation modeling helps to emphasize the iterative nature of model validation. The model actually becomes a form of working hypothesis, guiding the laboratory work and being refined in response to it.

## DISCUSSION

The obvious complexity of the carcinogenic process underscores the need for models that balance necessary detail with purposeful simplification. The available data suggest that the PCM achieves this balance. Moolgavkar et al. (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981; Moolgavkar et al., 1980) have shown that a biologically based two-stage model is sufficient to model human cancer incidence and to provide insight to the consequences of different carcinogenic mechanisms. The physiologically-based description of carcinogen pharmacokinetics works well for a variety of compounds (Bischoff and Brown, 1966; Matthews and Dedrick, 1984; Ramsey and Andersen, 1984; Andersen et al., 1987). The mechanisms described for DNA-damaging agents, promoters, and cytotoxicants are well-supported by the literature; however, they are not, by any means, the only such mechanisms that could be modeled. Although the model incorporates this substantial level of biological detail, it is still tractable. Tumor prevalence curves for two years of intermittent exposure (Figure 6.1-5) can be generated in about 15 min of VAX 8500 cpu time. Dose-

response curves for two years of continuous exposure (Figure 6.1-6) take about three minutes each. Many aspects of model behavior also can be readily studied with microcomputers.

Much, but not all, of what is known about the biology of chemical carcinogenesis is encoded in the PCM. The immune system, for example, is not explicitly described and may kill initiated and malignant cells (Thor et al., 1977). At present, any effect of the immune system on tumor prevalence is accounted for implicitly by $a_1$, $\beta_1$, $\mu_1$, and $\mu_2$. Genetic recombination appears to be an important mechanism for some cancers (Friedberg, 1985) but is not described. The current version of the model could not be validated properly for such agents. It has been shown that age-related changes in percent body fat affect pharmacokinetic behavior (Lutz et al., 1977). This finding suggests that, for a constant external concentration of carcinogen, the target tissue concentration will change with time. Therefore, for quantitatively accurate simulations, the PCM will need to describe the aging of its encoded biological structure. Each of these factors, which is either missing from the PCM or described implicitly, could be explicitly described and would fit into the pre-existing structure. Cell killing mediated by the immune system could be described as a first-order process acting on initiated and malignant cells. In the current deterministic version of the PCM, genetic recombination could be described as a process increasing $\mu_2$ as has been described by Mcolgavkar (1986). The model could be revised to exhibit age-related changes in body composition and biochemical processes simply by inserting tables of age-specific values for the affected parameters.

The ease with which foreseeable changes in the PCM fit into its pre-existing structure are characteristic of biologically based simulation modeling. Computer programs are, by their nature, easy to change. Because the model explicitly describes biological structure, future insights into chemical carcinogenesis will doubtless lead to additions and refinements but almost certainly not to fundamental changes in model structure.

Each element of the PCM (Figure 6.1-1) has been described thoroughly by other investigators. This work is innovative because it blends previously separate elements. When combined with computer simulation, this integration provides a flexible tool to use for step-by-step examination of the process of chemical carcinogenesis and, with validation, for rationally estimating the risks of exposure to chemical carcinogens.

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## 6.2 QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP MODELING OF CHEMICAL METABOLISM

### P. Seybold[a] and R. Conolly

### INTRODUCTION

The development of physiologically-based pharmacokinetic (FB-PK) models to predict the uptake, distribution, metabolism, and elimination of chemicals has been of interest for a number of years. Parameters necessary for model development include physiological constants, tissue solubilities, and metabolic constants. The required physiological parameters have been obtained from the open literature, but suitable tissue solubilities and metabolic constants require direct experimentation that is time-consuming and technically demanding. In an effort to reduce the need for direct experimentation of these two chemical-specific parameters, an attempt is being made to develop quantitative structural activity relationships (QSAR). This would, ultimately, allow the necessary chemical parameters to be calculated without need for laboratory experiments.

### RESEARCH PLAN

The goal of this research is to employ theoretical methods to examine the role of molecular structural features in halocarbon metabolism. The main experimental data base consists of enzyme kinetic rate data for various halogenated hydrocarbons. The theoretical techniques of choice for this study are quantum chemical molecular orbital calculations. The initial phases of the project encompassed three major areas: (1) installation and testing of appropriate molecular orbital computer programs, (2) review of the theoretical approaches related to the metabolism of halocarbons, (3) theoretical studies of metabolic activation related to the possible carcinogenic activities of these compounds. Of the theoretical approaches examined, the most useful were judged to be the semi-empirical AM1 method of Dewar et al. (1985) for general studies and higher-level ab initio calculations for selected, specific applications. Review of the relevant experimental literature is ongoing.

### RESULTS

In the present stage, theoretical calculations on the parent halocarbon compounds and possible early intermediates are being conducted. Optimized geometries for 25 parent compounds were obtained using the AM1 method. Comparison with experimental gas phase structures (mostly from electron-diffraction and microwave experiments) for 18 of these compounds shows generally good correlation between the theoretical and experimental results, suggesting that the

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method is sufficiently accurate for these purposes. At present, calculations are being conducted on biradical oxygenated species that are possible models for transition states of the initial metabolism of the haloethylenes by the cytochrome P-450 enzymes. Among the aspects of interest are the influence of halogen position on    initial site of oxygen attack and the influence of the nature of the halogen (Cl or Br) on the reaction rate.

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## SECTION 7

## AIR FORCE CHEMICAL DEFENSE TOXICOLOGY

### 7.1 PHARMACOKINETICS AND PHARMACODYNAMICS OF DIISOPROPYLFLUOROPHOSHATE

J. Cramer, S. Hsu, and J. Gearhart

### INTRODUCTION

A physiologically-based pharmacokinetic and pharmacodynamic model for the *in vivo* behavior of organophosphates (OP) is being developed by the Air Force to use in risk assessment and management of civilian and military exposures. The model can simulate the time course of the organophosphate diisopropylfluorophoshate (DFP) concentrations, in blood and brain, in conjunction with the inhibition of OP-susceptible esterases, such as acetylcholinesterase and carboxylesterase.

The objective of this research effort is to collect pharmacokinetic and pharmacodynamic data in rats exposed to DFP by inhalation, gavage, and dermal routes to validate the OP model.

### MATERIALS AND METHODS

#### Determination of a $LD_{50}$ for DFP

To establish nonlethal exposure levels for *in vivo* testing, an $LD_{50}$ for DFP was determined. Fischer-344 rats, weighing approximately 210 g, were dosed in groups of six animals per group, by injection via the femoral artery. The animals were purchased from Charles River (Kingston, NY) at weights of 125 to 150 g. Upon receipt from the vendors, the animals were housed in plastic cages with wood-chip absorbent bedding. All rats were fed Purina Formulab #5008 feed and softened water *ad libitum*. After quarantine and quality control procedures, the animals were available for experimentation.

DFP was obtained from Sigma and checked for hydrolysis products by GC. The chromatograph oven and injector temperatures were set at 100° and 200°C. The flame ionization detector (FID) temperature was 250°C, and the column was a nonpolar 10% SP2100, 80/100 Supelcoport, 6-ft long with a 1/8-in. diameter.

For intravenous dosing, the neat material was diluted with normal saline to achieve the necessary concentrations. The doses of DFP ranged from 2.048 to 0.852 mg/kg body weight.

*Determination of Air-Tissue Partition Coefficents*

To determine the optimum time of incubation for DFP with the particular tissue of interest, DFP was incubated in saline from 3 min to 3 h and the headspace concentration vs. time was plotted. The optimum incubation time for any air-tissue in mixture in this study was determined to be 70 min.

To determine the air-tissue partition coefficient for the selected tissues, the tissue material that DFP will partition into (i.e., saline, blood, or homogenized tissue) is placed in a liquid scintillation vial. Ten microliters of DFP was delivered with a microsyringe through a Teflon septum at the top of the vial onto the surface of the test material. The vial was then placed into a shaking heater block at 37°C until equilibrium was reached (~70 min). After equilibrium has been achieved, a gas-tight syringe is used to extract a headspace sample for GC analysis.

## RESULTS AND DISCUSSION

Table 7.1-1 depicts the experimental results from the intravenous administration of DFP. The $LD_{50}$ for DFP by intravenous administration was determined to be 1.75 mg/kg of body weight. The $LD_{50}$ in rats for other routes of exposure for this toxic compound are 6 mg/kg by oral gavage, 1.28 mg/kg by intraperitoneal injection, and 1.82 mg/kg by intramuscular injection (RETECS, 1977). Although the $LD_{50}$ of DFP determined in this study was below the oral and intramuscular routes of exposure, as expected, it was not below the intraperitoneally injected dose. With compounds as toxic as DFP, the steepness of the dose-response curve makes the exact evaluation of the $LD_{50}$ difficult. The nearly all-or-none response between the doses of 1.45 and 1.748 mg/kg, which is characteristic of potent toxics, exhibits this fact.

**TABLE 7.1-1. $LD_{50}$ DETERMINATION FOR DFP IN RATS**

| Dose (mg DFP/kg) | No. Dead/ Total Treated | % Mortality |
|---|---|---|
| 0 | 0/6 | 0 |
| 0.852 | 0/6 | 0 |
| 1.150 | 0/6 | 0 |
| 1.450 | 0/6 | 0 |
| 1.748 | 4/6 | 67 |
| 2.048 | 6/6 | 100 |

The tissue-air partition coefficients for DFP are provided in Table 7.1-2 . These experimental data presently are being used to further develop a physiologically-based pharmacokinetic model for organophosphates. To validate this model, it will be necessary to conduct studies on the

## TABLE 7.1-2. PARTITION COEFFICIENTS OF DFP

| Tissue | Partition Coefficients |
|---|---|
| Blood/Air | 12.57 |
| Liver/Blood | 1.53 |
| Kidney/Blood | 1.63 |
| Fat/Blood | 17.60 |
| Slowly Perfused/Blood | 0.77 |
| Brain/Blood | 0.67 |
| Richly Perfused/Blood | 0.67 |

pharmacokinetics of DFP. Efforts are presently being directed to determine the kinetics of DFP in rats.

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# SECTION 8

## STUDIES ON CW AGENT SIMULANTS

### 8.1 MODELING AND MEASUREMENT OF EXPIRED CHLOROPENTAFLUOROBENZE AFTER AN INHALATION EXPOSURE

A. Vinegar and D. Winsett

#### INTRODUCTION

Jepson et al. (1985) reported a procedure for evaluating candidate chemical agent uptake simulants. The first compounds studied were the perfluorocarbons, chosen because of their good detectability and relative biological inertness. Partition coefficients were determined for eight of these compounds using a modification of a vial equilibration technique described by Sato and Nakajima (1979). Metabolic constants were determined using a closed chamber gas uptake system described by Gargas et al. (1986).

The partition coefficients and metabolic data were used in a computer simulation of the chemical exposure. The model developed was physiologically-based and written in ACSL (Advanced Continuous Simulation Language) (Ramsey and Andersen, 1983). Metabolism could be described as a single saturable process, a first order process, or a combination of the two. The saturable process has a maximum metabolic rate ($V_{max}$, mg/h), and a Michaelis constant (Km, mg/L). The units of the first order constant (kfc) are $h^{-1}$. Metabolic constants ($V_{max}$, Km, and kfc) are varied in the model to fit the experimental data. The set of constants that fits the data over a range of exposure concentrations then can be used to assess the metabolism of the chemical being studied.

Of the eight compounds, chloropentafluorobenzene (CPFB) was selected for further study because of its good detectability, acceptable physical properties, and relative biological inertness. There were no deaths resulting from a 4-h exposure of rats to 4.84 mg/L of CPFB (Kinkead et al., 1987).

Based on the above findings, the study reported here was conducted to predict and measure the concentration of CPFB vapor expired by Fischer-344 rats after a 1-h, nose-only exposure to the compound. Also, the interaction of data collection with model development and the eventual improvement of the model resulting from the interaction are demonstrated.

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## MATERIALS AND METHODS

### Chemical

Chloropentafluorobenzene was obtained from Aldrich Chemical Company, Inc., at 95% purity. Analysis was confirmed by GC/MS determination.

### Vapor Generating and Exposure System

Filtered air was pumped though a nebulizer containing liquid CPFB. The nebulizer was maintained at 50°C in a water bath. Vapor passed though a second nebulizer to remove any aerosol, which may have formed, and was diluted with a second stream of filtered air. The diluted vapor stream was passed though a four-port, nose-only exposure chamber (volume = 815.6 cm$^3$). Vapor was pulled from the chamber and vented to a hood housing the whole system. A second stream of vapor was pulled though a GC sampling valve with a 1-mL sample loop to allow on-line analysis by GC (HP5880A, flame ionization detector, column: 20" x 1/8" SS, 2% OV101, 100-120 Chomosorb W) of the chamber concentration of CPFB (1 sample/min). The flow though the chamber averaged 0.73 L/min, and the chamber pressure was maintained slightly negative (-0.8 cm $H_2O$) by balancing the inlet and outlet flows. The animal was exposed by placing it into a restrainer tube, which plugged into one of the ports of the exposure chamber.

### Expired Air Analysis

The animal restrainer tube was disconnected from the exposure chamber and plugged into a manifold which provided a flow of filtered room air by the nose of the animal. The rate of flow (0.8 L/min) was selected to be greater than peak expiratory flow during normal tidal breathing. Thus, the animal would not rebreathe vapors it had exhaled. A side-stream sample of the now diluted expired air was pulled though (100 mL/min) the GC sample loop. Samples were taken from this side stream for 1 h post-CPFB exposure at intervals of 1/0.62 min for about 20 min and then 1/min for the rest of the hour.

### Minute Ventilation

Minute ventilation was monitored during the hour of exposure and the hour of breath analysis postexposure. The restrainer tube was configured as an integrated flow plethysmograph using a design similar to Mauderly et al. (1986); however, the flow element was a 400-mesh screen built into the wall of the tube rather than a separate pneumotachograph. Design considerations for the plethysmograph are outlined by Sinnett et al. (1981). The pressure drop across the plethysmograph flow element was detected with a Validyne DP45 2-cm $H_2O$ pressure transducer. This signal, conditioned by an HP3805B carrier demodulator, was calibrated as a flow integrated by

an HP8815A respiratory integrator and produced a second signal that was calibrated and recorded as minute ventilation on an HP7758A recorder.

## General Procedure

Eighteen male Fischer-344 rats were divided into groups of six. All animals were placed into the restrainer tube for a 2-h acclimation period two days before an experiment. On the day prior to an experiment, the animals were put though a training procedure protocol being exposed only to filtered air. On the day of the experiment they weighed 158.7 ± 15.6 g (mean ± standard deviation). The actual studies consisted of 1-h exposures followed by 1-h measurements of expired breath. Each of the three groups was exposed to either 300, 600, or 1200 ppm of CPFB.

## Modeling

The physiologically-based pharmacokinetic model written for CPFB was used to simulate exposure to 300, 600, and 1200 ppm of this chemical and to predict the expected expired concentrations during 1-h postexposure.

## RESULTS AND DISCUSSION

Actual concentrations of CPFB measured during the postexposure period are shown in Figure 8.1-1. Results shown are for one animal at 300 ppm CPFB and are plotted with the simulated data for an animal of the same body weight. Ventilation (QP) and cardiac output (QC) were set as equal constants for the simulation as they are calculated allometrically using the equations $QP = QC = 14M^{0.7}$, where M is body mass in kg. The continuous line represents the simulation of the concentration of CPFB measured in parts per million during the hour of exposure and the hour of postexposure. The data points plotted during the hour postexposure are the actual experimental measurements. It is apparent that the fit of the simulation to the actual data is not very good. The actual data are higher than the simulated values. The trend over time is similar, but the actual data are scattered when compared with the simulation.

Minute ventilation, measured thoughout the experiment, was obviously not as constant as the model assumed. Under conditions of restraint, the animals were inhaling volumes greater than those predicted for the resting conditions assumed by the original model. Actual values for minute ventilation were inserted into the model in tabular form rather than using the allometrically determined value for ventilation. Clearly, if ventilation is increased, cardiac output is also increased and, to a large extent, can be expected to follow ventilation. The model, as indicated earlier, assumed that ventilation and cardiac output were equal. It has been determined that cardiac output is probably about half of ventilation when determined allometrically from body weight (Calder, 1984). To optimize the relationship of cardiac output to ventilation, the original closed chamber gas uptake data of Jepson et al. (1985) were reevaluated using Simusolv, an optimizer for programs written in ACSL. Optimization was performed for the first-order metabolic rate constant (KFC),

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cardiac output as a fraction of ventilation (QCFQP), and the coefficient for calculating ventilation (QPC). In this simulation, ventilation was kept constant and determined as an allometric function of body weight, but QPC was optimized. Ventilation had to be kept constant because it was not measured during the closed chamber gas uptake determinations. Figure 8.1-2 shows the original data collected during the gas uptake experiments of Jepson et al. (1985) coplotted with their observed fit of the simulation to the data. Each line and associated data points represent the uptake of three rats from a 9 1-L chamber containing starting concentrations of CPFB at 2137, 855, 214, and 10 parts per million. The values for KFC, QCFQP, and QPC used in this simulation were 2, 1, and 14, respectively. Using Simusolv to optimize the fit resulted in values for KFC, QCFQP, and QPC of 4, 0.6, and 19, respectively. The optimized simulated fit to the data is shown in Figure 8.1-3.

Figure 8.1-4 shows the fit when the optimized values for KFC and QCFQP obtained from Jepson et al. (1985) are used in the simulations of the current experiments with the actual ventilation values for each animal. The simulation now follows the actual measured concentrations of CPFB during the postexposure period. The example shown for one animal at 300 ppm is representative of what was observed for all animals at all three concentrations. These simulations represent the best approximation to the actual data collected within the limits of those parameters that were chosen to be measured and of those chosen to be optimized. An even better simulation would result if the exact relationship between cardiac output and ventilation were known over the whole range of metabolic activity of an animal.

The interaction of the modeling process with the experimental data collection resulted in a model that is a good predictor of expired concentrations of CPFB. Parameters were optimized, and with the additional measurement of ventilation, the model will follow the shorter term fluctuations in expired concentrations. Notice that the model simulates the expired concentration during the exposure and postexposure periods.

Having made two noninvasive measurements, expired concentrations of CPFB and ventilation, and using the actual ventilatory data in this model, which is physiologically-based (i.e., it accounts for blood flows to and from organ systems and for the partitioning of CPFB between blood and the organ systems), several predictions can be made. These are as follows.

1. total amount of CPFB inhaled,

2. total amount of CPFB exhaled,

3. total dose reaching different organ systems,

4. concentration changes over time in these systems, and

5. cardiac output over time.

Thus, the initial effort of developing and validating a physiologically-based model can provide access to less readily measured parameters, such as the dose of a chemical reaching an organ system.

**Figure 8.1-1. Concentration of CPFB Measured in Expired Breath During and After an Inhalation Exposure to 300 ppm.** Time on the abscissa is for an hour of exposure followed by an hour postexposure. CMPPM on the ordinate is the concentration measured in parts per million. The continuous line represents the simulated expired concentration, and the pluses represent the actual measurements during the hour postexposure.
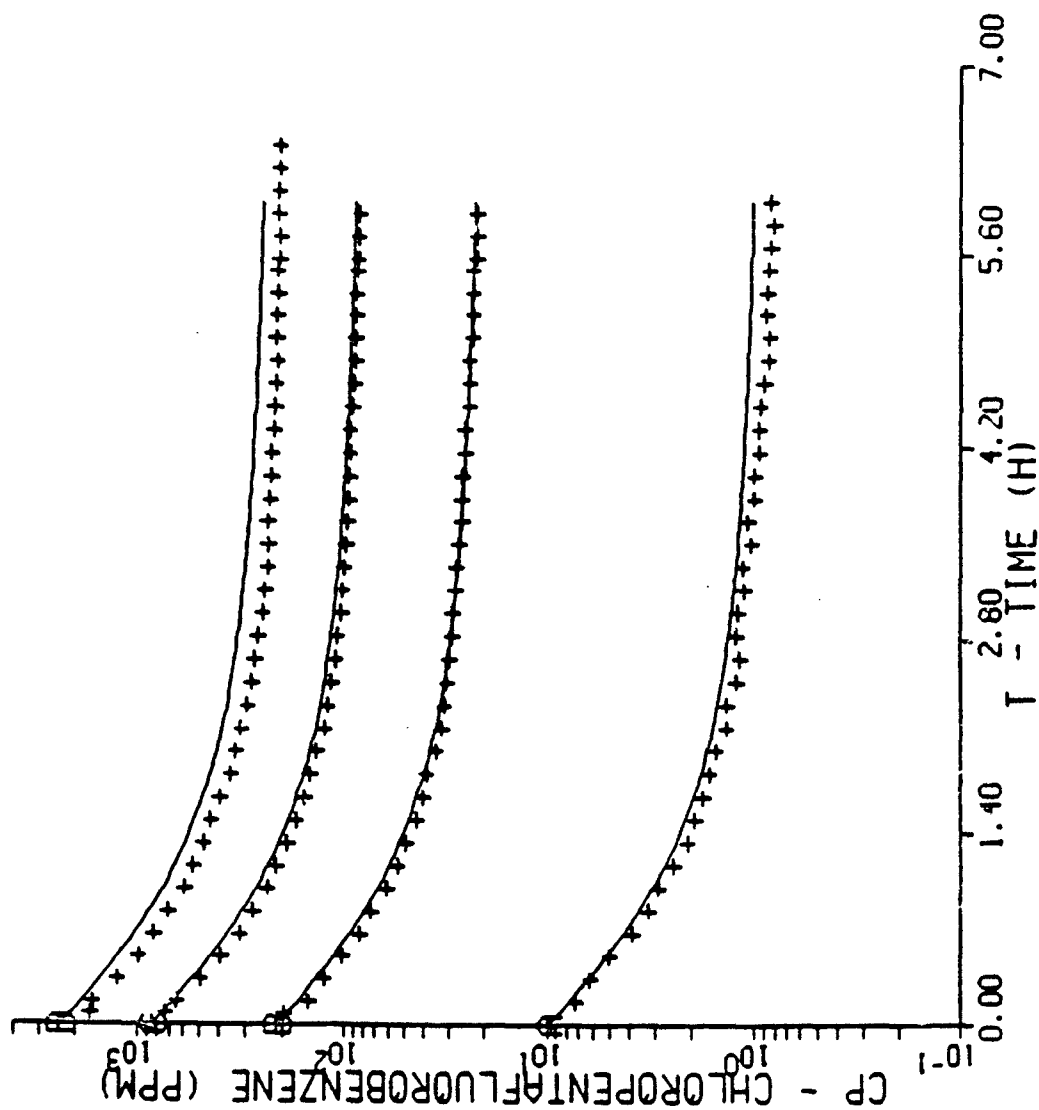
Figure 8.1-2. Concentration of CPFB Measured in a Closed Chamber Containing Three Rats. Initial concentrations were 2137, 855, 214, and 10 ppm. Pluses represent the actual concentrations over the 6- to 7-h measurement period. The continuous lines are the simulation (from Jepson et al., 1985).
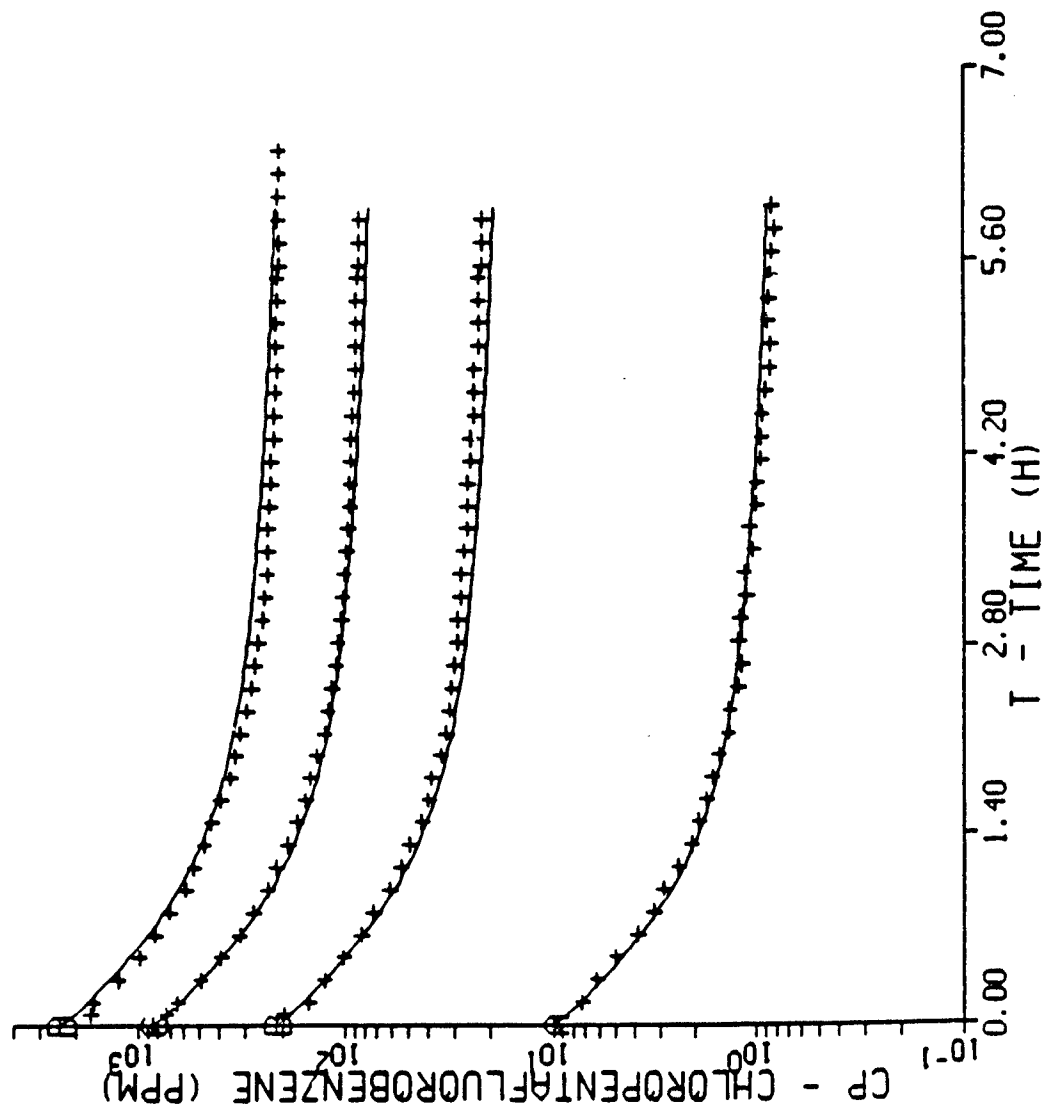
Figure 8.1-3. Concentration of CPFB Measured in a Closed Chamber Containing Three Rats. As in Figure 8.1-2, Except the Simulation was Optimized for the First-Order Metabolic Rate Constant (kfc), the Ratio of Cardiac Output to Ventilation (QCFQP) and the Ventilatory Constant (QPC).
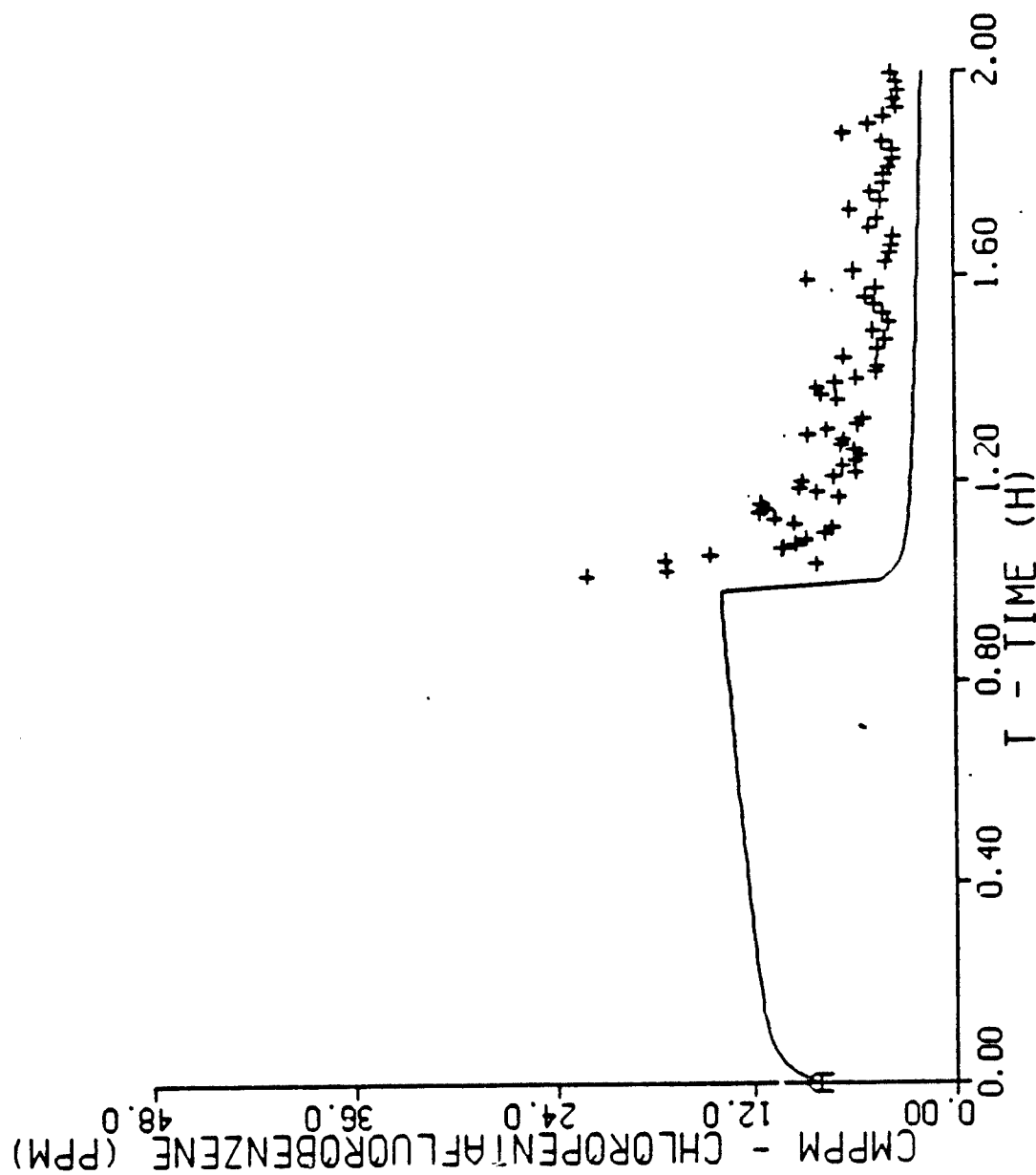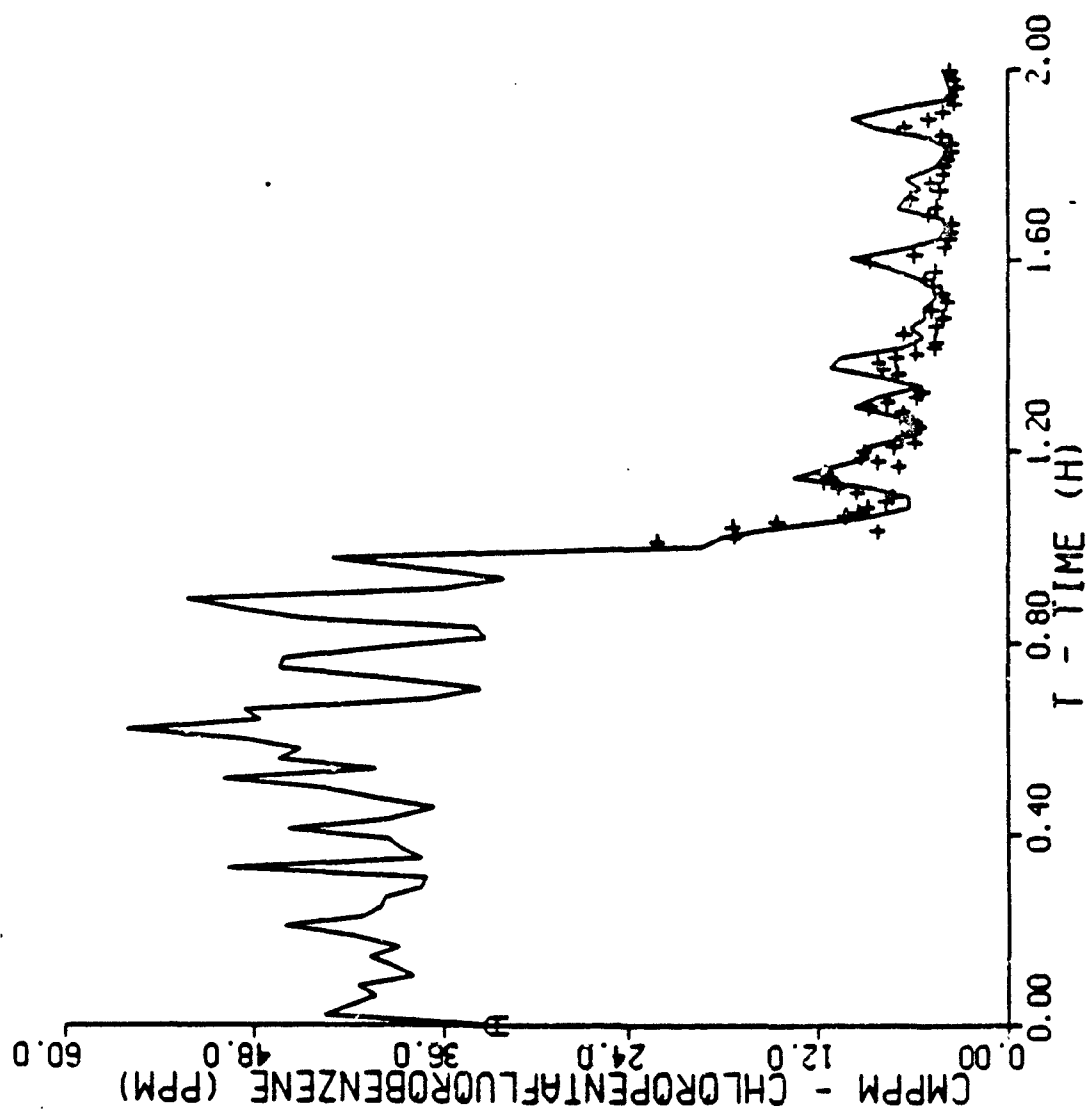
Figure 8.1-4. Concentration of CPFB Measured in Expired Breath During and After an Inhalation Exposure to 320 ppm. As in Figure 8.1-1, Except the Optimized Values of Kfc and QCFQP Were Used Along With the Actual Ventilatory Data.

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## 8.2 *IN VITRO* STUDIES INVESTIGATING THE GENOTOXIC POTENTIAL OF CHLOROPENTAFLUOROBENZENE

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### INTRODUCTION

The biological activity of chloropentafluorobenzene (CPFB) was evaluated using six short-term *in vitro* assays. CPFB, a fully substituted halogenated benzene, is a volatile, colorless liquid being considered by the Air Force to test the effectiveness of chemical defense procedures and equipment. CPFB is appropriate because it is detectable at very low levels by gas chromatography (GC). However, little is known about the potential human health hazards associated with the exposure to this agent. To estimate the health risks of CPFB, *in vitro* assays were performed to assess the ability of this agent to damage DNA and/or to transform mammalian cells. The assays were chosen to cover a broad range of different types of DNA damage and to compare results with previous assay data (Tu et al., 1986). These studies were performed to assess the mutation, DNA repair, cytogenetic abnormalities, and transformation rates of cells exposed to CPFB. Because little is known about the possible requirement for metabolic activation of CPFB, the studies were performed both without and with metabolic activation. Metabolic activation was accomplished by adding rat liver microsomes to the exposure mixture (Zeiger et al., 1979).

The studies reported here were conducted in glass culture vessels. Initial studies were conducted to determine the solubility of CPFB in culture media (Kutzman, 1987) and to investigate its reported potential to cloud conventional plastic culture dishes (Tu et al., 1986). Corroboration of this report and the greater solubility of CPFB in the absence of plastic supported the rationale for conducting these studies in glass dishes.

### MATERIALS AND METHODS

#### Test Compound

In the first phase of these studies, the CPFB stock material was analyzed for identity and purity. One 100-mL bottle of CPFB (lot# MM03511TH) was purchased from Aldrich Chemical Company (Cat. No. 19,366). The bottle was labeled 95% pure, F.W. 202.51, $ClC_6F_5$, b.p. 122-123°C/750 mm Hg,

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and density 1.568 g/mL. The test sample was stored at 4°C in a locked refrigerator, and the refrigerator temperature was monitored daily and recorded weekly. The positive control chemicals, 4-nitro-quinoline-1-oxide (4NQO), sodium azide, 2-nitrofluorene, and 2-aminoanthracene, were purchased from Sigma Chemical Co. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz(a)anthracene (DMBA) were obtained from the National Toxicology Program (NTP) repository. All positive control compounds were kept at -20°C in a locked freezer, and the freezer temperature was monitored daily and recorded weekly.

Quantitative chemical analysis of the CPFB sample using quadrapolar mass spectrometry (MS) and GC revealed that the compound was >99.7% pure. The impurities were not identified further.

## Preparation of Glass Dishes for Cell Culture

Clean glass dishes were treated by boiling them in a solution of 10 mM ethylene-diaminetetraacetic acid (EDTA) for 10 min. The dishes were then sterilized by autoclaving for 30 min.

## Metabolic Activation

To simulate in vitro any possible metabolic activation of CPFB that might occur in vivo, duplicate assays were performed using a rat liver homogenate rich in metabolizing enzymes. For this purpose, Aroclor 1254-induced rat liver microsomal fraction (S9), lot number R-289, was purchased from Microbiological Associates, Inc. The "S9 mix" used for metabolic activation consisted of 6 mL S9, 40 mM nicotinamide adenine dinucleotide phosphate (NADP), 50 mM glucose-6-phosphate, and 330 mM KCl in 15 mL of culture medium. This was diluted further by adding 1 part "S9 mix" to 19 parts culture medium before use. This activation system was used in the Salmonella Mutagenicity Assay, Sister Chromatid Exchange (SCE) Assay, Chromosome Aberration (CAb) Assay and the BALB/c Transformation Assay. A modified activation system was used for the Chinese Hamster Ovary (CHO) Cell Mutation Assay as described below.

## Salmonella Mutagenicity Assay

The Salmonella Mutagenicity Assay used a methodology similar to that described by Ames et al. (1973,1975). Cytotoxicity of CPFB was determined using the Salmonella typhimurium TA98 and TA100 strains in the plate incorporation assay. This cytotoxicity assay was performed by treating one-day-old bacterial cultures with 0.10, 0.33, 1.0, 3.3, or 10 μL of CPFB in 2 mL of molten agar (45°C). This agar, which contained trace amounts of histidine and biotin, was then poured over minimal medium-E agar plates (GIBCO). The plates were incubated at 37°C for 48 h, and the colonies were counted with an Artek automatic colony counter Model 980 (New Brunswick Scientific). This process was followed by a mutagenicity assay with TA98 and TA100 strains, conducted in a nontoxic dose

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range. Metabolic activation was incorporated into the mutagenic assay by adding a post-mitochondrial supernatant fraction from rat livers induced with Aroclor 1254. The tester strains used in these studies were designed to detect frameshift mutagens (TA1537, TA1538, and TA98) and base pair substitutions (TA1535 and TA100). The strains were verified for the expression of phenotypic characteristics (his-, rfa, uvrB, and ampR) and response to concurrent positive controls. The CPFB was diluted in DMSO. The auxotrophic strains of *Salmonella typhimurium* were obtained from Dr. B.N. Ames, University of California, Berkeley, CA.

For the mutagenicity assays, overnight cultures of *Salmonella* tester strains (TA1535, TA1537, TA1538, TA98, and TA100) were incubated with the test sample in a nontoxic dose range (0.1 to 10 µL per plate) and tested in triplicate at five dose levels in borosilicate glass tubes. Agar (2 mL) containing trace amounts of histidine and biotin was then added (45°C). The mixture was swirled on a low speed vortex mixer and poured over previously prepared minimal medium-E plates. The plates were incubated at 37°C for 72 h, and the revertant colonies were counted on an Artek automatic colony counter.

The data were interpreted as follows. Either a mutagenic ratio of 2.0 or more, or a consistent and dose-related increase in revertant induction, was considered a positive response. Comparisons were made with spontaneous reversion counts as well as with historical reversion counts. Experiments were considered valid if the spontaneous counts and the positive control data were similar to the historical data (Claxton et al., 1982; Maron and Ames, 1983).

### Mammalian Cell Mutation Assay

CHO cells were used to determine the ability of CPFB to mutate mammalian DNA. The CHO cell line is a permanent cell line with an average generation time of 12 to 14 h. The cells were grown at 37°C in a water-saturated atmosphere of 5% $CO_2$. The cells were maintained in McCoy's 5A medium (GiBCO) supplemented with 10% FBS, 2 µM L-glutamine, and 1% gentamycin (Gibco). The cultures were subcultured once- or twice-a-week at a cell concentration that allowed them to be maintained as logarithmically growing cultures. The cultures were either passaged or refed twice weekly. Cultures were plated for subculture at $5 \times 10^5$ cells/100-mm glass dish. Cultures were handled under gold lights to prevent phototoxic effects. The culture conditions were rigorously controlled to maintain a relatively uniform cloning efficiency and growth rate as well as a low spontaneous mutation frequency. The cultures were trypsinized for subculture by first rinsing with calcium- and magnesium-free phosphate buffered saline (PBS), then adding 3 mL of 0.2% trypsin-EDTA. A single-cell suspension was used for subculture by making the appropriate dilutions ($5 \times 10^5$ cells/100 mm-glass dish) following counting. The cells were counted using a Coulter cell counter.

The cytotoxic effects of CPFB on CHO cells were assessed using a relative cloning efficiency (RCE) assay, which measured the reduction in colony-forming ability of the cells. For this assay, the cells were plated at $2 \times 10^2$ cells/100-mm glass dish 24 h prior to chemical treatment. The cells were then exposed for 24 h to 0.1, 1, 10, 100, and 1000 μg of CPFB per mL, washed twice with buffered saline, and refed with fresh medium. In the presence of metabolic activation, the exposure duration was limited to 2 h. The cells were then incubated in the culture medium described above for 7 days. At the end of the incubation period, the culture dishes were rinsed with buffered saline and fixed with methylene blue (0.2%) in 30% methanol. The number of colonies containing 50 or more cells were counted for each dish using an Artek automatic colony counter. The RCE and the surviving fraction were calculated from the number of colonies observed in the solvent control and treatment conditions. Nontoxic concentrations of CPFB to be used in the mutation assays were determined using the RCE data.

The same lot number of liver homogenate (S9 fraction) was used throughout the experimental procedure and was stored at -80°C. A modified activation system was used in this assay for optimal performance. The S9 fraction was thawed immediately before use and mixed to give the following final reaction mixture in the culture medium: 2.4 mg NADP (sodium salt)/mL, 4.5 mg isocitric acid (trisodium salt)/mL, and 15 μL S9 fraction/mL.

On the day after subculture, replicate log-phase cultures in glass 100-mm culture dishes were treated with growth medium containing selected concentrations of CPFB or a positive control chemical, either MNNG or B(a)P. In assays without metabolic activation, CPFB treatment lasted 24 h and MNNG treatment lasted 4 h, after which time the medium containing CPFB or the control chemical was removed and fresh culture medium without additional chemical was added. In assays with metabolic activation, CPFB and B(a)P treatments lasted 2 h. Twenty-four hours after the initiation of chemical treatment, the cells were replated and their viability determined by plating $2 \times 10^2$ cells from each test or control culture into 10 mL of medium in 100-mm plastic dishes. The dishes were incubated for 7 days, and the number of colonies per dish was determined as in the cytotoxicity assay.

The presence of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) mutants was determined by plating at least $10^6$ cells from each test chemical treatment or control group into 10 or more dishes containing 10 μg 6-thioguanine (6-TG)/mL for a final cell density per culture dish of $1 \times 10^5$. The mutant selection was initiated at 7 to 9 days following the initiation of chemical treatment. At the time of selection initiation, a second survival assay was conducted similar to the one at 24 h. The selection cultures were maintained by refeeding them with selective medium on

Day 3, and incubation was continued for an additional 6 to 7 days. The cultures were then fixed and stained as in the cytotoxicity assay, and the colonies counted and recorded.

The mutation frequency was determined by dividing the total number of HGPRT (6-TG resistant) mutants scored by the number of viable cells plated (determined by multiplying the seeding density by the cloning efficiency for each test or control condition).

A mutation assay was considered valid and acceptable if

- the cloning efficiency of solvent control cultures was >60%,

- the mutation frequency of the positive control was similar to that expected based on historical data (O'Neill et al., 1977; Arce et al., 1987), and

- at least three of the test chemical concentrations had acceptable numbers of viable cells selected for mutation (at least $5 \times 10^5$).

A response to a test chemical was considered positive in the HGPRT mutation assay if

- there were reproducible increases in mutation frequency at two or more concentrations (a mutation frequency that is greater than 50 mutant colonies per $10^6$ plated cells may be considered as positive depending upon the solvent control frequency); or

- the number of mutant colonies present in the treatment dishes was greater than five times that observed in the solvent control for at least two of the treatment concentrations.

These criteria are not absolute, and final interpretation of data may be based on the previous experience of investigators in consideration of any extenuating circumstances.

The interpretation of data may consider the relative degree of response as well as existing data on other chemicals with known responses in the assay. Ultimately, the interpretation of the in vitro responses must consider the potential for in vivo exposure and other factors.


### Mammalian Cell Cytogenetic Assays

Two assays were used to assess the ability of CPFB to induce cytogenetic damage in CHO cells: the SCE Assay and the CAb Assay.


### Assay for Sister Chromatid Exchange

One day after culture initiation, the medium was removed and the CHO cells were treated for 2 h with 62.5, 125, 250, 500, and 1000 µg CPFB/mL of medium before adding bromodeoxyuridine (BuDr). BuDr was added to visualize the SCEs within the DNA. Following a 2-h exposure to CPFB, 0.1 µg BuDr/mL was added to the cultures. The cells were incubated for 24 h. The medium was then removed, fresh medium containing BuDr and colcemid (0.1 µg/mL) was added, and incubation

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continued for 2 to 3 h. The total incubation time with CPFB was 26 h. When metabolic activation was provided, the medium on one-day-old cultures was replaced with S9 mix in medium plus the test chemical without FBS. In such tests, the CPFB and S9 were present for only the initial 2 h.

### Cell Harvest and Fixation

Two to three hours following addition of colcemid, the cells were collected by mitotic shake-off and treated for up to 3 min at room temperature with hypotonic KCl (75 mM). Cells were then washed twice with fixative (3:1, methanol:glacial acetic acid, v/v), dropped onto slides, and air dried.

### Assay for Chromosome Aberrations

The CAb Assay was performed in a manner similar to the SCE test except that no BuDr was used. In treatments without S9 mix, the cultures were incubated with CPFB for 8 to 10 h, washed, and treated with colcemid for 2 to 2.5 h before fixation. In tests involving activation, CPFB and S9 mix were present for only the initial 2 h.

The cells from the SCE and CAb studies were selected for scoring on the basis of normal morphology and a chromosome number of $21 \pm 3$. A modified fluorescence plus Giemsa technique was used to stain the cells for the presence of SCE in the second metaphase stage of replication. In this procedure, the slides were stained for 10 min with Hoechst 33258 (5 μg/mL) in phosphate buffer (pH 6.8), mounted in the same buffer, and exposed at 55 to 65°C to "black light" from 15-W tubes for 3 to 8 min. Finally, slides were stained with Giemsa and air-dried. These cells were then scored for the frequency of SCE per cell. For scoring aberrations, slides were stained with Giemsa, and individual types of aberrations were scored separately. Fifty cells per treatment group were scored in each assay.

Data analysis of both SCEs and aberrations was developed by the National Institute of Environmental Health Sciences (NIEHS)/NTP and was employed in the CPFB study. This data analysis uses a decision scheme that considers both a trend analysis of the dose-response curve and the magnitude of the response at each dose level. A repeat of each test is required, and the results of two tests are then used to assign a negative, questionable, or positive conclusion. In the assays performed here using CPFB, the Student's t-test was used to examine the differences between the treated and control groups.

### Primary Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay

Hepatocytes were isolated from male Fischer 344 rats using the two-step perfusion method of Seigen (1973). Freshly isolated rat hepatocytes were plated onto standard glass slides in 100-mm glass dishes. The cells were cultured in EMEM supplemented with 0.2 mM serine, 0.2 mM aspartate, 1 mM pyruvate, 5% FBS, 0.1 μM insulin, and 50 μg gentamycin/mL. Cell viability was determined

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using trypan blue. Following a 2-h attachment period, the cells were exposed to CPFB in media, 4-nitroquinilone-1-oxide (4NQO) in DMSO (final DMSO concentration was 0.2% in medium), 0.2% DMSO in medium alone, or medium alone for 18 h in a humidified incubator. During this period, the cells were also exposed to 10 µCi tritiated thymidine (specific activity: 88 Ci/mmole)/mL. The cells were rinsed with FBS, 1% sodium citrate was added to the dishes for 15 min, then the cells were fixed in methanol and dried. The glass slides and dishes were dipped in Kodak NTB2 liquid emulsion and exposed for 14 days at -20°C. The slides and dishes were photographically developed, fixed, and stained with Giemsa stain. The exposed grains in cells with intact nuclear membranes were counted at 1000X magnification using an Artek automatic colony counter with a video camera mounted on a standard Nikon microscope. The grain counts for 50 cells per experimental group were recorded. The mean and standard deviation were determined, and the Student's t-test was used to determine the significance between test means and control means (Simpson et al., 1960). Mean values were considered significantly different if $p < 0.001$. While there are no generally accepted statistical techniques for this assay, the $p < 0.001$ level of significance was used previously in similar studies (Tu et al., 1965).

### BALB/c-3T3 Transformation Assay

This transformation assay procedure and the criteria for scoring transformed foci in the BALB/c-3T3 cell culture system were published previously (Kakunaga, 1973; Reznikoff et al., 1973).

For the cytotoxicity assay, $2 \times 10^2$ cells were seeded in 60-mm dishes in 5 mL EMEM supplemented with 10% FBS. Three dishes were used for each treatment or control group. After the cells had attached (18 to 24 h), the cultures were treated with 0.1, 1, 10, 100, and 1000 µg CPFB/mL of complete medium for 72 h. Cells were then washed twice with PBS and fed with fresh EMEM supplemented with 10% FBS. Seven days later, the cultures were fixed and stained. Colonies containing 50 or more cells were counted, and the RCE was determined.

For the transformation assay, $3 \times 10^4$ 3T3 cells were seeded into 60-mm dishes in EMEM with 10% FBS 24 h before treatment. Cultures were then treated with 0.1, 1, 10, 100, and 1000 µg of CPFB per mL of EMEM with 10% FBS. The positive control plates were treated with 0.1 and 0.25 µg B(a)P/mL. B(a)P was dissolved initially in DMSO then added to the culture medium so that the final DMSO concentration was 0.2% (v/v). Following exposure for 72 h, the cultures were washed twice with PBS and refed with EMEM and 10% FBS medium. The medium was replaced twice a week for 24 days, at which time the cultures were fixed and stained with Giemsa. Control cultures were handled in the same manner except that DMSO alone was added to a final concentration in the medium of 0.2% (v/v). Both type II and type III foci were counted, but only type III foci were used to estimate transformation rates. The transformation rates were determined using two methods: the

Mean Method as developed by Capizzi and Jameson (1973) and the $P_0$ Fluctuation Analysis Method of Luria and Delbruck (1943). Transformation frequencies were correlated with relative cellular survival and calculated as described by Reznikoff et al. (1973). For each concentration of CPFB or control group, 12 dishes were used in the transformation assay and three additional dishes were used to confirm the cytotoxicity at that concentration.

### Statistical Methodology

There is a wide variance in statistical techniques used for analyzing data from in vitro assays. Several criteria were developed for each assay to aid in analyzing the data (Auletta, 1985). The data in this report were analyzed by two methods: empirical methods (e.g., a two- to threefold increase compared to background indicates a positive response for most mutation assays) and the Student's t-test (Simpson et al., 1960). The statistical analyses are not corrected for multiple comparisons to a single experimental control.

### RESULTS

Salmonella Mutagenicity Assay

The toxicity data from experiments where Salmonella bacteria were exposed to CPFB are given in Table 8.2-1. Neither strain, TA98 or TA100, exhibited significant (>50%) toxicity when exposed to 0.1 to 10 µL CPFB per plate. From this data, it is assumed that CPFB is not cytotoxic to all five Salmonella strains up to a maximum dose of 10 µL per plate.

Table 8.2-2 illustrates the mutagenicity data with tester strains TA1535, TA1537, TA1538, TA98, and TA100. CPFB was not mutagenic when tested at concentrations up to 10 µL per plate. These data suggest that CPFB is devoid of mutagenic activity (both frameshift and base pair substitution types) in the Salmonella assay when tested up to a maximum dose of 10 µL per plate. Incorporation of S9 metabolic activation had no effect on this result.

#### TABLE 8.2-1. CYTOTOXICITY OF CPFB TO SALMONELLA STRAINS

| CPFB[c] (uL/plate) | Average No. Revertants[a] | | % Survival[b] | |
|---|---|---|---|---|
| | TA98 | TA100 | TA98 | TA100 |
| 10.0 | 18.33 | 114.00 | 63% | 87% |
| 3.30 | 18.00 | 123.33 | 62% | 95% |
| 1.00 | 15.33 | 120.00 | 53% | 92% |
| 0.33 | 22.00 | 140.33 | 76% | 108% |
| 0.10 | 25.33 | 112.00 | 91% | 86% |
| Media Control | 29.00 | 130.33 | 100% | 100% |

[a] An average of three counts.
[b] Percent of control.
[c] There were 2 mL of CPFB-containing top agar per plate. CPFB has a density of 1.568 g/mL. Therefore, 10 µL CPFB/plate would be equivalent to 7.84 µg CPFB/mL of agar.

## TABLE 8.2-2. MUTAGENIC ACTIVITY OF CPFB IN THE AMES ASSAY

| CPFB (µL)/plate[b] | 10% S9[c] | Revertants per plate[a] | | | | |
|---|---|---|---|---|---|---|
| | | TA1535 | TA1537 | TA1538 | TA98 | TA100 |
| Media Control | − | 37 | 23 | 21 | 40 | 71 |
| 0.10 | − | 22 | 23 | 22 | 31 | 69 |
| 0.33 | − | 26 | 15 | 22 | 31 | 88 |
| 1.00 | − | 22 | 18 | 26 | 27 | 42 |
| 3.33 | − | 25 | 21 | 24 | 39 | 75 |
| 10.00 | − | 32 | 18 | 24 | 37 | 73 |
| | | | | | | |
| Media Control | + | 24 | 25 | 42 | 55 | 119 |
| 0.10 | + | 11 | 40 | 30 | 37 | 93 |
| 0.33 | + | 21 | 17 | 31 | 40 | 89 |
| 1.00 | + | 13 | 19 | 32 | 40 | 66 |
| 3.33 | + | 17 | 21 | 42 | 49 | 62 |
| 10.00 | + | 21 | 26 | 46 | 62 | 85 |
| | | | | | | |
| Positive Controls | | | | | | |
| Sodium azide (3 µg/plate) | − | 204 | − | − | − | 1069 |
| 2-Nitrofluorene (10 µg/plate) | − | − | 112 | 747 | 399 | − |
| 2-aminoanthracene (10 µg/plate) | + | 293 | 263 | 1441 | 1795 | 824 |

[a] An average of six plate counts. Those plates indicating a positive mutagenic response are underlined.
[b] There were 2 mL of CPFB-containing top agar per plate. CPFB has a density of 1.568 g/mL. Therefore, 10 µL CPFB/plate would be equivalent to 7.84 µg CPFB/mL of agar.
[c] S9 = metabolic activation mixture.

### Mammalian Cell Mutation Assay

The cytotoxicity of the CPFB was determined in preliminary CHO mammalian cell assays. Concentrations of CPFB up to 1000 µg/mL produced no obvious cytotoxicity in the cloning efficiency assays with CHO cells. The mutagenicity data for CPFB in the absence of exogeneous metabolic activation are presented in Tables 8.2-3 and 8.2-4. From these data it is obvious that CPFB did not induce mutation at the HGPRT locus in CHO cells. The induced mutation frequency observed at 500 µg CPFB/mL did appear to be reproducible with 5.4- and 4.2-fold increases in the two tests, respectively, compared to control. The mutation induction with the positive control, MNNG, was at the expected value (greater than 500-fold increase compared to control). There were no solubility problems in these assays. The mutagenicity data for CPFB in the presence of exogenous activation (S9) are presented in Tables 8.2-5 and 8.2-6. The B(a)P concentration produced the expected response in the assay. This concentration was intentionally selected to ensure that the S9 activation system was sufficient to activate a concentration of B(a)P that was known to produce a weak but positive response. There was no positive activity with CPFB in this HGPRT mutation assay with CHO cells, according to the criteria for a positive response as stated in the Materials and Methods Section. Thus, from these data, CPFB appears to be inactive in the HGPRT/CHO Mutation Assay both with and without exogeneous metabolic activation.

#### TABLE 8.2-3. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION – EXPERIMENT 1

| Concentration (µg/mL) | Average No. Mutants per 100,000 Cells | Relative Survival | Induced Mutation Frequency per Million Cells | Fold Increase over Background |
|---|---|---|---|---|
| Media Control | 1.3 | 1 | 13 | 1 |
| MNNG | | | | |
| 0.5 | 113.5 | 0.02 | 58,121.46 | 4,470.88 |
| CPFB | | | | |
| 1,000 | 5.68 | 1.82 | 31.19 | 2.40 |
| 500 | 7.40 | 1.05 | 70.61 | 5.43 |
| 250 | 1.50 | 1.34 | 11.16 | 0.86 |
| 125 | 1.35 | 1.23 | 10.94 | 0.84 |
| 62.5 | 1.00 | 1.07 | 9.37 | 0.72 |

#### TABLE 8.2-4. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION – EXPERIMENT 2

| Concentration (µg/mL) | Average No. Mutants per 100,000 Cells | Relative Survival | Induced Mutation Frequency per Million Cells | Fold Increase over Background |
|---|---|---|---|---|
| Media Control | 0.47 | 1.00 | 4.67 | 1.00 |
| MNNG | | | | |
| 1.0 | 5.80 | 0.01 | 5,355.33 | 1,146.75 |
| 0.5 | 108.47 | 0.17 | 6,259.43 | 1,340.35 |
| CPFB | | | | |
| 1,000 | 0.27 | 1.21 | 2.20 | 0.47 |
| 500 | 2.07 | 1.05 | 19.61 | 4.20 |
| 250 | 0.40 | 1.16 | 3.44 | 0.74 |
| 125 | 0.20 | 1.28 | 1.56 | 0.33 |
| 62.5 | 0.67 | 1.18 | 5.65 | 1.21 |

#### TABLE 8.2-5. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION – EXPERIMENT 3

| Concentration (µg/mL) | Average No. Mutants per 100,000 Cells | Relative Survival | Induced Mutation Frequency per Million Cells | Fold Increase over Background |
|---|---|---|---|---|
| Media Control | 1.40 | 1.00 | 14.00 | 1.00 |
| B(a)P | | | | |
| 1.0 | 2.50 | 0.93 | 26.86 | 1.92 |
| CPFB | | | | |
| 1,000 | 1.10 | 0.52 | 21.26 | 1.52 |
| 500 | 1.00 | 0.67 | 14.85 | 1.06 |
| 250 | 0.55 | 0.43 | 12.86 | 0.92 |
| 125 | 0.90 | 0.75 | 12.07 | 0.86 |
| 62.5 | 0.80 | 0.58 | 13.70 | 0.98 |

**TABLE 8.2-6. EFFECT OF CPFB ON THE MUTATION FREQUENCY OF
CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION – EXPERIMENT 4**

| Concentration (µg/mL) | Average No. Mutants per 100,000 Cells | Relative Survival | Induced Mutation Frequency per Million Cells | Fold Increase over Background |
|---|---|---|---|---|
| Media Control | 0.40 | 1.00 | 4.00 | 1.00 |
| B(a)P | | | | |
| 1.0 | 2.10 | 0.89 | 23.71 | 5.93 |
| CPFB | | | | |
| 1,000 | 0.42 | 0.61 | 6.92 | 1.73 |
| 500 | 0.57 | 0.63 | 9.03 | 2.26 |
| 250 | 0.35 | 0.95 | 8.90 | 2.23 |
| 125 | 0.45 | 0.54 | 8.30 | 2.08 |
| 62.5 | 0.16 | 0.70 | 2.24 | 0.56 |

### Mammalian Cell Cytogenetic Assays

CPFB induced positive results in four tests using the SCE Assay (Tables 8.2-7 to 8.2-10). In all experiments, 1000 µg CPFB/mL induced significant numbers of SCEs, comparable to those induced by the positive control carcinogen DMBA. In the two experiments using exogenous metabolic activation, SCEs were also induced in significant numbers at concentrations of 1000 µg CPFB/mL (Tables 8.2-9 and 8.2-10). There was no appreciable cell-cycle delay at the highest doses of CPFB scored with and without activation. CPFB induced SCEs in a dose-dependent manner with and without activation.

CPFB induced marginally significant numbers of CAbs at a concentration of 1000 µg/mL in two experiments without metabolic activation (Tables 8.2-11 and 8.2-12). In those experiments using metabolic activation, CPFB again induced marginally significant numbers of CAbs at 1000 and 500 µg/mL in both experiments (Tables 8.2-13 and 8.2-14); however, the level of significance at 500 µg/mL for one of these experiments (Table 8.2-14) was only $p < 0.05$. Nonetheless, a dose-related increase in CAbs was observed in the assays with and without metabolic activation.

TABLE 8.2-7. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITHOUT METABOLIC ACTIVATION – EXPERIMENT 1

| Concentration (µg/ml) | Cell Kinetic Index[a] | Total No. Chromosomes Scored | Total No. SCEs Scored | Sum of SCEs per Chromosome[b] | Mean No. Chromosomes Per Cell ± SD | Mean No. SCEs Per Cell ± SD | Mean No. SCEs per Chromosome ± SD[c] |
|---|---|---|---|---|---|---|---|
| Media Control | 2.00 | 1,074 | 100 | 5.02 | 19.89 ± 1.12 | 1.85 ± 0.85 | 0.093 ± 0.042 |
| DMSO (0.2%) | 1.98 | 1,017 | 89 | 4.53 | 19.4 ± 0.84 | 1.71 ± 0.93 | 0.087 ± 0.046 |
| DMBA | | | | | | | |
| 1.0 | 2.03 | 658 | 216 | 10.89 | 19.9 ± 1.10 | 6.55 ± 3.99 | 0.330 ± 0.209[***d] |
| 0.1 | 2.10 | 880 | 264 | 13.44 | 19.6 ± 0.78 | 5.87 ± 2.79 | 0.299 ± 0.139[****] |
| CPFB | | | | | | | |
| 1000 | 2.02 | 1,236 | 333 | 16.95 | 19.6 ± 0.82 | 5.29 ± 2.50 | 0.269 ± 0.128[***] |
| 500 | 2.03 | 1,489 | 295 | 15.07 | 19.6 ± 1.04 | 3.88 ± 1.82 | 0.198 ± 0.092[*] |
| 250 | 2.02 | 1,644 | 344 | 17.38 | 19.8 ± 0.98 | 4.14 ± 1.69 | 0.209 ± 0.085[*] |
| 125 | 2.00 | 1,421 | 261 | 13.09 | 19.7 ± 1.17 | 3.63 ± 2.03 | 0.182 ± 0.095 |
| 62.5 | 2.00 | 1,560 | 188 | 9.40 | 20.0 ± 1.11 | 2.41 ± 1.88 | 0.120 ± 0.093 |

[a] Cell kinetic index = (first division cells + 2 × second division cells + 3 × third division cells) + N, where N equals the number of cells scored (Schneider et al., 1981).
[b] Approximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell.
[c] Mean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored.
[d] Significantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control).

* p < 0.05
** p < 0.01
*** p < 0.005
**** p < 0.001

TABLE 8.2-8. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITHOUT METABOLIC ACTIVATION – EXPERIMENT 2

| Concentration (μg/mL) | Cell Kinetic Index[a] | Total No. Chromosomes Scored | Total No. SCEs Scored | Sum of SCEs per Chromosome[b] | Mean No. Chromosomes Per Cell ± SD | Mean No. SCEs Per Cell ± SD | Mean No. SCEs per Chromosomes ± SD[c] |
|---|---|---|---|---|---|---|---|
| None | 1.98 | 1,012 | 69 | 3.50 | 19.8 ± 1.05 | 1.35 ± 0.71 | 0.069 ± 0.037 |
| DMSO (0.2%) | 1.96 | 1,011 | 72 | 3.58 | 20.2 ± 1.22 | 1.44 ± 0.70 | 0.072 ± 0.035 |
| DMBA | | | | | | | |
| 1.0 | 1.98 | 1,006 | 269 | 13.67 | 19.7 ± 0.95 | 5.27 ± 1.73 | 0.268 ± 0.088****[d] |
| 0.1 | 1.98 | 1,011 | 210 | 10.64 | 19.8 ± 0.86 | 4.12 ± 2.12 | 0.209 ± 0.110* |
| CPFB | | | | | | | |
| 1000 | 2.02 | 1,045 | 326 | 16.33 | 20.1 ± 1.33 | 6.27 ± 2.26 | 0.314 ± 0.118**** |
| 500 | 1.98 | 1,037 | 197 | 9.91 | 19.9 ± 1.32 | 3.79 ± 1.31 | 0.191 ± 0.066** |
| 250 | 1.98 | 975 | 199 | 10.03 | 19.9 ± 0.95 | 4.06 ± 1.42 | 0.204 ± 0.073*** |
| 125 | 2.00 | 1,044 | 203 | 10.09 | 20.1 ± 1.30 | 3.90 ± 1.67 | 0.194 ± 0.081* |
| 62.5 | 1.93 | 458 | 64 | 3.21 | 19.9 ± 1.02 | 2.78 ± 0.78 | 0.139 ± 0.038 |

[a] Cell kinetic index = (first division cells + 2 x second division cells + 3 x third division cells) ÷ N, where N equals the number of cells scored (Schneider et al., 1981).
[b] Approximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell.
[c] Mean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored.
[d] Significantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control).

* $p < 0.05$
** $p < 0.01$
*** $p < 0.005$
**** $p < 0.001$

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TABLE 8.2-9. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITH METABOLIC ACTIVATION – EXPERIMENT 3

| Concentration (μg/mL) | Cell Kinetic Index[a] | Total No. Chromosomes Scored | Total No. SCEs Scored | Sum of SCEs per Chromosome[b] | Mean No. Chromosomes Per Cell ± SD | Mean No. SCEs Per Cell ± SD | Mean No. SCEs per Chromosome ± SD[c] |
|---|---|---|---|---|---|---|---|
| None | 1.96 | 1,015 | 55 | 2.78 | 19.9 ± 0.95 | 1.08 ± 0.68 | 0.054 ± 0.035 |
| DMSO (0.2%) | 1.96 | 1,023 | 62 | 3.10 | 20.1 ± 1.19 | 0.06 ± 0.05 | 0.060 ± 0.025 |
| DMBA | | | | | | | |
| 1.0 | 1.96 | 1,011 | 262 | 13.24 | 19.8 ± 0.98 | 5.14 ± 1.57 | 0.260 ± 0.084****d |
| 0.1 | 1.96 | 995 | 190 | 9.55 | 19.9 ± 0.85 | 3.80 ± 2.02 | 0.191 ± 0.101* |
| CPFB | | | | | | | |
| 1000 | 2.06 | 1,051 | 315 | 15.70 | 20.2 ± 1.36 | 6.05 ± 2.17 | 0.302 ± 0.112**** |
| 500 | 1.96 | 1,018 | 191 | 9.62 | 20.0 ± 1.34 | 3.75 ± 1.39 | 0.169 ± 0.072*** |
| 250 | 1.95 | 973 | 205 | 10.33 | 19.9 ± 0.95 | 4.18 ± 1.64 | 0.211 ± 0.082*** |
| 125 | 1.98 | 1026 | 196 | 9.78 | 20.1 ± 1.17 | 3.84 ± 1.54 | 0.192 ± 0.077*** |
| 62.5 | 1.98 | 999 | 120 | 6.00 | 20.0 ± 1.16 | 2.40 ± 0.89 | 0.120 ± 0.044 |

a Cell kinetic index = (first division cells + 2 x second division cells + 3 x third division cells) ÷ N, where N equals the number of cells scored (Schneider et al., 1981).
b Approximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell.
c Mean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored.
d Significantly different from respective controls (DMBA vs DMSO, CPFB vs. Media Control)

* p < 0.05
** p < 0.01
*** p < 0.005
**** p < 0.001

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TABLE 8.2-10. EFFECT OF CPFB ON THE INDUCTION OF SCEs WITH METABOLIC ACTIVATION – EXPERIMENT 4

| Concentration (µg/mL) | Cell Kinetic Index [a] | Total No. Chromosomes Scored | Total No. SCEs Scored | Sum of SCEs per Chromosome [b] | Mean No. Chromosomes Per Cell ± SD | Mean No. SCEs Per Cell ± SD | Mean No. SCEs per Chromosome ± SD [c] |
|---|---|---|---|---|---|---|---|
| Media Control | 1.98 | 1,035 | 85 | 4.27 | 19.9 ± 1.04 | 1.63 ± 0.94 | 0.082 ± 0.047 |
| DMSO (0.2%) | 1.96 | 1,008 | 91 | 4.57 | 20.2 ± 1.25 | 1.82 ± 1.05 | 0.091 ± 0.055 |
| DMBA | | | | | | | |
| 1.0 | 1.96 | 1,011 | 266 | 13.47 | 19.8 ± 1.00 | 5.22 ± 1.70 | 0.264 ± 0.086****[d] |
| 0.1 | 1.96 | 989 | 212 | 10.77 | 19.8 ± 0.86 | 4.24 ± 2.06 | 0.215 ± 0.106*[c] |
| CPFB | | | | | | | |
| 1000 | 1.98 | 1,031 | 275 | 13.76 | 20.2 ± 1.35 | 5.39 ± 2.88 | 0.270 ± 0.149***[c] |
| 500 | 1.98 | 1,019 | 178 | 8.98 | 20.0 ± 1.29 | 3.49 ± 1.42 | 0.176 ± 0.074 |
| 250 | 1.98 | 1,018 | 186 | 9.31 | 20.0 ± 0.91 | 3.65 ± 1.57 | 0.102 ± 0.077* |
| 125 | 2.00 | 1,041 | 149 | 7.44 | 20.0 ± 1.23 | 2.87 ± 1.86 | 0.143 ± 0.091 |
| 62.5 | 1.96 | 1,003 | 121 | 6.06 | 20.1 ± 1.17 | 2.42 ± 1.44 | 0.121 ± 0.073 |

[a] Cell kinetic index = (first division cells + 2 x second division cells + 3 x third division cells) ÷ N, where N equals the number of cells scored.
[b] Approximately 50 cells scored per concentration for the SCEs. Sum of the SCEs per chromosome determined for each cell.
[c] Mean number of SCEs per chromosome is derived by dividing the sum of SCEs per chromosome by the number of cells scored.
[d] Significantly different from respective controls (DMBA vs DMSO, CPFB vs. Media Control).

* $p < 0.05$
** $p < 0.01$
*** $p < 0.005$
**** $p < 0.001$

TABLE 8.2-11. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITHOUT METABOLIC
ACTIVATION – EXPERIMENT 1

| Concentration (µg/mL) | Number of Cells Analyzed | % of Cells Showing | | | | | |
|---|---|---|---|---|---|---|---|
| | | Chromatid Gaps[a] | Chromatid Aberrations[b] | Chromosome Aberrations[c] | Multiple Aberrations[d] | All Aberrations Excluding Gaps[e] | All Aberrations Including Gaps[f] |
| Media Control | 300 | 1.3 | 0 | 0 | 0 | 0 | 1.3 |
| DMSO (0.2%) | 300 | 9.0 | 0 | 1.0 | 0 | 1.0 | 10.0 |
| DMBA | | | | | | | |
| 1.0 | 94 | 2.0 | 23.0 | 33.0 | 0 | 56.0***[g] | 58.0 |
| 0.1 | 188 | 8.0 | 10.0 | 6.0 | 0 | 16.0*** | 24.0 |
| CPFB | | | | | | | |
| 1000 | 300 | 4.3 | 2.0 | 3.0 | 0 | 5.0** | 9.3 |
| 500 | 300 | 3.0 | 1.0 | 0.5 | 0 | 1.5 | 4.5 |
| 250 | 300 | 1.0 | 1.0 | 1.0 | 0 | 2.0 | 3.0 |
| 125 | 300 | 2.0 | 1.5 | 0 | 0 | 1.5 | 3.5 |
| 62.5 | 300 | 1.0 | 0.5 | 1.0 | 0 | 1.5 | 2.5 |

[a] Gaps and/or isogaps.
[b] Breaks or single fragments or exchange figures or acentric rings or chromatid minutes.
[c] Acentric fragments or dicentrics or translocations or rings or minutes.
[d] Any combination of identifiable chromatid or chromosome aberrations.
[e] All types of chromatid and chromosome-type aberrations excluding chromatid gaps.
[f] All types of chromatid and chromosome-type aberrations including chromatid gaps.
[g] Significantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control).

   * $p < 0.05$
  ** $p < 0.01$
  *** $p < 0.001$

TABLE 8.2-12. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITHOUT
METABOLIC ACTIVATION – EXPERIMENT 2

| Concentration (µg/mL) | Number of Cells Analyzed | % of Cells Showing | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Chromatid Gaps[a] | Chromatid Aberrations[b] | Chromosome Aberrations[c] | Multiple Aberrations[d] | All Aberrations Excluding Gaps[e] | All Aberrations Including Gaps[f] |
| Media Control | 300 | 0 | 0 | 1 | 0 | 1 | 1 |
| DMSO (0.2%) | 300 | 2 | 0 | 0 | 0 | 0 | 2 |
| DMBA | | | | | | | |
| 1.0 | 300 | 4.0 | 12.0 | 11.0 | 0 | 23.0[***][g] | 27.0 |
| 0.1 | 300 | 6.0 | 6.0 | 4.0 | 3 | 10.0[**] | 16.0 |
| CPFB | | | | | | | |
| 1000 | 300 | 3.6 | 1.6 | 4.0 | 0 | 5.6[**] | 9.2 |
| 500 | 300 | 3.3 | 0.6 | 2.6 | 0 | 3.2 | 6.5 |
| 250 | 303 | 2.3 | 0.6 | 1.3 | 0 | 1.9 | 4.2 |
| 125 | 300 | 0.5 | 0.5 | 0.5 | 0 | 1.0 | 1.5 |
| 62.5 | 300 | 1.0 | 0 | 0.5 | 0 | 0.5 | 1.5 |

[a] Gaps and/or isogaps.
[b] Breaks or single fragments or exchange figures or acentric rings on chromatid minutes.
[c] Acentric fragments or dicentrics or translocations or rings or minutes.
[d] Any combination of identifiable chromatid or chromosome aberrations.
[e] All types of chromatid and chromosome-type aberrations excluding chromatid gaps.
[f] All types of chromatid and chromosome-type aberrations including chromatid gaps.
[g] Significantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control).
  * p<0.05
  ** p<0.01
  *** p<0.001

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## TABLE 8.2-13. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITH METABOLIC ACTIVATION – EXPERIMENT 3

| Concentration (µg/mL) | Number of Cells Analyzed | % of Cells Showing | | | | | |
|---|---|---|---|---|---|---|---|
| | | Chromatid Gaps[a] | Chromatid Aberrations[b] | Chromosome Aberrations[c] | Multiple Aberrations[d] | All Aberrations Excluding Gaps[e] | All Aberrations Including Gaps[f] |
| Media Control | 300 | 1.0 | 0 | 0 | 0 | 0 | 1.0 |
| DMSO (0.2%) | 200 | 1.5 | 0 | 0 | 0 | 0 | 1.5 |
| DMBA | | | | | | | |
| 1.0 | 200 | 11.5 | 20.0 | 33.0 | 0 | 53.0***g | 64.5 |
| 0.1 | 85 | 18.0 | 10.0 | 19.0 | 0 | 29.0** | 47.0 |
| CPFB | | | | | | | |
| 1000 | 300 | 5.3 | 1.3 | 3.3 | 0 | 4.6** | 9.9 |
| 500 | 300 | 6.0 | 2.3 | 1.0 | 0 | 3.3** | 9.3 |
| 250 | 300 | 3.0 | 0.6 | 1.0 | 0 | 1.6 | 4.6 |
| 125 | 300 | 2.0 | 0 | 1.5 | 0 | 1.5 | 3.5 |
| 62.5 | 300 | 1.0 | 0 | 1.0 | 0 | 1.0 | 2.0 |

[a] Gaps and/or isogaps.
[b] Breaks or single fragments or exchange figures or acentric rings on chromatid minutes.
[c] Acentric fragments or dicentrics or translocations or rings or minutes.
[d] Any combination of identifiable chromatid or chromosome aberrations.
[e] All types of chromatid and chromosome-type aberrations excluding chromatid gaps.
[f] All types of chromatid and chromosome-type aberrations including chromatid gaps.
[g] Significantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control).
  * p < 0.05
  ** p < 0.01
  *** p < 0.001

## TABLE 8.2-14. EFFECT OF CPFB ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS WITH METABOLIC ACTIVATION – EXPERIMENT 4

| Concentration (µg/mL) | Number of Cells Analyzed | % of Cells Showing | | | | | |
|---|---|---|---|---|---|---|---|
| | | Chromatid Gaps[a] | Chromatid Aberrations[b] | Chromosome Aberrations[c] | Multiple Aberrations[d] | All Aberrations Excluding Gaps[e] | All Aberrations Including Gaps[f] |
| Media Control | 200 | 0.5 | 0 | 0 | 0 | 0 | 0.5 |
| DMSO (0.2%) | 300 | 1.5 | 0 | 0 | 0 | 0 | 1.5 |
| DMBA | | | | | | | |
| 1.0 | 300 | 16.0 | 13.0 | 19.0 | 0 | 32.0***g | 48.0 |
| 0.1 | 200 | 7.0 | 8.0 | 17.0 | 0 | 25.0** | 32.0 |
| CPFB | | | | | | | |
| 1000 | 200 | 5.0 | 1.5 | 3.0 | 0 | 4.5** | 9.5 |
| 500 | 300 | 3.0 | 1.0 | 1.3 | 0 | 2.3* | 5.3 |
| 250 | 300 | 2.0 | 1.3 | 1.0 | 0 | 2.3* | 4.3 |
| 125 | 300 | 1.5 | 1.0 | 0 | 0 | 1.0 | 2.5 |
| 62.5 | 300 | 0 | 0 | 0.4 | 0 | 0.4 | 0.4 |

a Gaps and/or isogaps.
b Breaks or single fragments or exchange figures or acentric rings on chromatid minutes.
c Acentric fragments or dicentrics or translocations or rings or minutes.
d Any combination of identifiable chromatid or chromosome aberrations.
e All types of chromatid and chromosome-type aberrations excluding chromatid gaps.
f All types of chromatid and chromosome-type aberrations including chromatid gaps.
g Significantly different from respective controls (DMBA vs. DMSO, CPFB vs. Media Control):
  * $p < 0.05$
  ** $p < 0.01$
  *** $p < 0.001$

*Primary Rat Hepatocyte UDS Assay*

The cytotoxic effects of CPFB were determined by trypan blue exclusion following treatment. The cells were exposed to concentrations of CPFB ranging from 62.5 to 1000 µg/mL. Viability of the primary rat liver cells was above 60% for all doses used. The data from the first experiment showed no significant evidence of unscheduled DNA synthesis (DNA repair from damage) at any dose of CPFB (Table 8.2-15). The net number of grains per nucleus, analyzed in cells of the solvent and media control groups, was typically a negative number (McQueen and Williams, 1985). The values of the positive controls were significantly higher than solvent controls. In cells exposed to the highest concentration of CPFB (1000 µg/mL), there were slight but not significant increases in UDS. Also, at such a high concentration, osmolality effects, pH effects, and other artifacts could cause DNA damage. The second experiment (Table 8.2-16) confirmed the results of the first experiment. These results show that concentrations of CPFB up to 1000 µg/mL produce no significant DNA damage as detected by unscheduled DNA synthesis in primary rat hepatocytes.

TABLE 8.2-15. EFFECT OF CPFB ON PRIMARY RAT HEPATOCYTE
UNSCHEDULED DNA SYNTHESIS – EXPERIMENT 1

| Concentration (µg/mL) | Net Grains Per Nucleus[a] | |
|---|---|---|
| | Mean | Standard Deviation |
| Media Control | -0.24 | 0.76 |
| DMSO (0.2%) | -0.30 | 1.04 |
| 4NQO | | |
| 1.0 | 9.64[b] | 4.62 |
| 0.1 | 1.62[b] | 2.15 |
| CPFB | | |
| 1000 | 0.16 | 1.03 |
| 500 | -0.30 | 0.81 |
| 250 | -0.32 | 1.09 |
| 125 | -0.10 | 1.35 |
| 62.5 | -0.20 | 1.13 |

[a]Average of 50 cells per group. Net grains/nucleus = grains/nucleus – grains/ cytoplasm (equal area).
[b]Significantly different from DMSO controls at $p < 0.001$.

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**TABLE 8.2-16. EFFECT OF CPFB ON PRIMARY RAT HEPATOCYTE UNSCHEDULED DNA SYNTHESIS – EXPERIMENT 2**

| Concentration (µg/mL) | Net Grains Per Nucleus[a] | |
|---|---|---|
| | Mean | Standard Deviation |
| Media Control | -0.54 | 1.28 |
| DMSO (0.2%) | -0.14 | 0.85 |
| 4NQO | | |
| 1.0 | 4.65[b] | 3.64 |
| 0.1 | 0.58[b] | 1.43 |
| CPFB | | |
| 1000 | 0.10 | 1.59 |
| 500 | -0.30 | 1.37 |
| 250 | -0.24 | 1.52 |
| 125 | -0.14 | 1.36 |
| 52.5 | -0.16 | 1.75 |

[a]Average of 50 cells per group. Net grains/nucleus = grains/nucleus – grains/cytoplasm (equal area).
[b]Significantly different from DMSO controls at $p < 0.001$.

### BALB/c-3T3 Transformation Assay

The cytotoxicity of CPFB using BALB/c-3T3 cells was determined by relative cloning efficiency. The cells were exposed to concentrations of CPFB from 0.1 to 1000 µg/mL. Transformation assays were also performed using this concentration range. The transformation data from the first experiment are presented in Table 8.2-17. CPFB was tested without the addition of metabolic activation, and the experiment was performed in 60-mm glass dishes. In this experiment, the induction of the transformed phenotype with B(a)P was somewhat lower than expected (Arce et al., 1987). In this first experiment, the transformation frequencies and rates for 1000 µg/mL CPFB were about half of those determined for 0.25 µg B(a)P; comparable frequencies and rates were observed when compared with 0.1 µg/mL B(a)P.

Three technical difficulties were associated with the use of glass dishes in this assay: (1) The cells tended not to attach to the glass dishes as well as the plastic dishes; (2) the loosely attached cells tended to lift off the dish during refeeding; and (3) the dishes were cumbersome and awkward to handle. These problems, coupled with the lower than expected response from the positive control, prompted a reexamination of the transformation frequency on plastic dishes. The transformation data for CPFB without metabolic activation performed on plastic dishes are shown in Table 8.2-18. The B(a)P concentrations tested induced the expected response in the assay. In this second experiment, 1000 µg CPFB/mL induced transformation frequencies and rates that were about one-third of those observed for 0.25 µg B(a)P/mL. Again, these CPFB-induced frequencies and rates were comparable to those observed for 0.1 µg B(a)P/mL. Thus, the use of the plastic dishes did not appear to modulate the response of either B(a)P or CPFB in the transformation assay.

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TABLE 8.2-17 EFFECT OF C??? ON THE TRANSFORMATION OF BALB/c-3T3 CELLS WITHOU  
METABOLIC ACTIVATION — EXPERIMENT 1[a]

| Concentration (μg/mL) | | Number of Dishes | Number of Type III Foci | Number of Type III Foci/Dish | Estimated Transformation Frequency[b] (Transformants per Cell Plated) | Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by: | |
|---|---|---|---|---|---|---|---|
| | | | | | | Mean Method[c] | P₀ Method[d] |
| Media Control | | 9 | 0 | 0 | $3.67 \times 10^{-6}$ | $9.8 \times 10^{-8}$ | $4.65 \times 10^{-8}$ |
| DMSO | (0.2%) | 6 | 0 | 0 | $5.71 \times 10^{-5}$ | $1.6 \times 10^{-7}$ | $5.32 \times 10^{-8}$ |
| B(a)P | 0.25 | 9 | 7 | 0.78 | $5.78 \times 10^{-5}$ | $2.6 \times 10^{-7}$ | $1.21 \times 10^{-7}$ |
| | 0.10 | 9 | 5 | 0.56 | $2.61 \times 10^{-5}$ | $2.2 \times 10^{-7}$ | $2.31 \times 10^{-7}$ |
| CPFB | 1000 | 8 | 4 | 0.50 | $2.69 \times 10^{-5}$ | $2.1 \times 10^{-7}$ | $2.40 \times 10^{-7}$ |
| | 100 | 6 | 2 | 0.30 | $1.23 \times 10^{-5}$ | $1.9 \times 10^{-7}$ | $1.41 \times 10^{-7}$ |
| | 10 | 6 | 1 | 0.16 | $5.91 \times 10^{-6}$ | $1.6 \times 10^{-7}$ | $6.32 \times 10^{-8}$ |
| | 1.0 | 8 | 0 | 0 | $4.33 \times 10^{-6}$ | $1.1 \times 10^{-7}$ | $4.63 \times 10^{-8}$ |
| | 0.1 | 8 | 0 | 0 | $< 1.00 \times 10^{-7}$ | $1.1 \times 10^{-7}$ | $4.63 \times 10^{-8}$ |

[a] Experiment was performed on 60-mm glass dishes.  
[b] Transformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973).  
[c] Transformation rate calculated by the mean method according to Capizzi and Jameson (1973).  
[d] Transformation rate calculated by the P₀ method using the Luria-Delbruck fluctuation analysis (1943)

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TABLE 8.2-18. EFFECT OF CPFB ON THE TRANSFORMATION OF BALB/c-3T3 CELLS WITHOUT METABOLIC ACTIVATION – EXPERIMENT 2[a]

| Concentration (μg/mL) | | Number of Dishes | Number of Type III Foci | Number of Type III Foci/Dish | Estimated Transformation Frequency[b] (Transformants per Cell Plated) | Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by: | |
|---|---|---|---|---|---|---|---|
| | | | | | | Mean Method[c] | $P_o$ Method[d] |
| Media Control | | 9 | 0 | 0 | $3.67 \times 10^6$ | $9.8 \times 10^8$ | $4.66 \times 10^8$ |
| DMSO | (0.2%) | 10 | 0 | 0 | $3.35 \times 10^6$ | $8.8 \times 10^8$ | $3.65 \times 10^8$ |
| B(a)P | 0.25 | 9 | 12 | 1.33 | $9.85 \times 10^5$ | $3.6 \times 10^7$ | $7.62 \times 10^7$ |
| | 0.10 | 11 | 9 | 0.82 | $3.82 \times 10^5$ | $2.4 \times 10^7$ | $4.50 \times 10^7$ |
| CPFB | 1000 | 10 | 6 | 0.60 | $3.23 \times 10^5$ | $1.5 \times 10^7$ | $3.18 \times 10^7$ |
| | 500 | 12 | 8 | 0.67 | $2.75 \times 10^5$ | $2.1 \times 10^7$ | $3.81 \times 10^7$ |
| | 250 | 12 | 2 | 0.17 | $5.91 \times 10^6$ | $9.7 \times 10^8$ | $6.32 \times 10^8$ |
| | 125 | 10 | 2 | 0.20 | $6.92 \times 10^6$ | $1.2 \times 10^7$ | $7.73 \times 10^8$ |
| | 62.5 | 12 | 1 | 0.08 | $2.76 \times 10^6$ | $7.3 \times 10^8$ | $3.02 \times 10^8$ |

[a] Experiment was performed on 60-mm plastic dishes.
[b] Transformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973)
[c] Transformation rate calculated by the mean method according to Capizzi and Jameson (1973).
[d] Transformation rate calculated by the $P_o$ method using the Luria-Delbruck fluctuation analysis (1943)

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BAL3/c-3T3 cells are capable of providing the metabolic activation required to induce transformation by a diverse group of carcinogens (Cortesi et al., 1983). Nonetheless, CFFB was tested in this system in the presence of metabolic activation (S9 fraction). The transformation data for CPFB in the presence of exogenous activation are presented in Tables 3.2-19 and 3.2-20. In these experiments, exogenous activation did not appear to affect the induction of transformation by B(a)P. However, the transformation frequencies (Reznikoff, 1973) and rates induced by 1000 $\mu$g CPFB/mL were comparable to or exceeded the frequencies and rates induced by 0.25 $\mu$g B(a)P/mL. Using the $P_o$ method, a fluctuation analysis test (Luria and Delbruck, 1943), the calculated transformation rates were the same for 1000 $\mu$g CPFB /mL and 0.25 $\mu$g B(a)P/mL. At 500 $\mu$g CPFB/mL, the transformation frequency and rate determined by the Mean Method (Capizzi and Jameson, 1973) were slightly less than that observed for 0.1 $\mu$g B(a)P/mL, whereas the transformation rate determined by the $P_o$ method was higher. Results of the second experiment using metabolic activation were similar to those observed in the first experiment. In all transformation experiments, regardless of the methods employed to calculate frequencies or rates, a dose-dependent response induced by CPFB was observed, indicating biological activity in this system. Our results suggest that a possible enhanced transformation rate can be observed in BALB/c-3T3 cells with metabolic activation of CPFB.

## DISCUSSION

In these studies, the genotoxic potential of CPFB was determined using six standard in vitro assays: the Salmonella Mutation Assay or Ames Assay, the CHO Mutation Assay, the CHO CAb Assay, the CHO SCE Assay, the Rat Hepatocyte UDS Assay, and the BALB/c Transformation Assay.

Plastic dishes generally are used in these assays because their surface properties allow cells to attach and grow well. However, in this study, it was necessary to use glass dishes to examine the genotoxic effects of CPFB, because this chemical has dissolved standard plastic tissue culture dishes (Kutzman, 1987), possibly producing erroneous test results. Because there was no methodology to handle compounds that degrade plastic, substantial efforts were expended to adapt the assays to incorporate the use of a glass substratum for cell attachment and growth. These efforts will facilitate the proper examination of other compounds with similar properties.

CPFB did not induce mutations in the DNA of Salmonella typhimurium as measured by the Ames assay. The rationale for using a bacterial system for genetic damage was based on the premise that the chemical reactivity and stability of the DNA between prokaryotic and eukaryotic cells are essentially similar. Liver fractions containing metabolizing enzymes were used in an attempt to produce metabolic products of CPFB and examine the potential genotoxicity of these metabolites.

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## TABLE 8.2-19. EFFECT OF CPFB ON THE TRANSFORMATION OF BALB/c-3T3 CELLS WITH METABOLIC ACTIVATION – EXPERIMENT 3[a]

| Concentration (μg/mL) | Number of Dishes | Number of Type III Foci | Number of Type III Foci/Dish | Estimated Transformation Frequency[b] (Transformants per Cell Plated) | Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by: | |
|---|---|---|---|---|---|---|
| | | | | | Mean Method[c] | $P_o$ Method[d] |
| Media Control | 12 | 0 | 0 | $2.67 \times 10^{-6}$ | $7.3 \times 10^{-8}$ | $3.02 \times 10^{-7}$ |
| DMSO (0.2%) | 8 | 0 | 0 | $4.20 \times 10^{-6}$ | $1.1 \times 10^{-7}$ | $4.63 \times 10^{-8}$ |
| B(a)P 0.25 | 8 | 9 | 1.13 | $8.37 \times 10^{-5}$ | $3.4 \times 10^{-7}$ | $7.21 \times 10^{-7}$ |
| 0.12 | 7 | 6 | 0.86 | $4.01 \times 10^{-5}$ | $3.0 \times 10^{-7}$ | $4.34 \times 10^{-7}$ |
| CPFB 1000 | 8 | 13 | 1.63 | $8.76 \times 10^{-5}$ | $4.2 \times 10^{-7}$ | $7.21 \times 10^{-7}$ |
| 500 | 8 | 6 | 0.75 | $3.08 \times 10^{-5}$ | $2.6 \times 10^{-7}$ | $4.81 \times 10^{-7}$ |
| 250 | 10 | 0 | 0 | $3.48 \times 10^{-6}$ | $8.8 \times 10^{-8}$ | $3.65 \times 10^{-8}$ |
| 125 | 6 | 0 | 0 | $5.77 \times 10^{-6}$ | $1.5 \times 10^{-7}$ | $6.32 \times 10^{-8}$ |
| 62.5 | 7 | 0 | 0 | $4.93 \times 10^{-6}$ | $1.3 \times 10^{-7}$ | $5.34 \times 10^{-8}$ |

[a] Experiment was performed on 60-mm plastic dishes.
[b] Transformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973).
[c] Transformation rate calculated by the mean method according to Capizzi and Jameson (1973).
[d] Transformation rate calculated by the $P_o$ method using the Luria-Delbruck fluctuation analysis (1943)

TABLE 8.2-20. EFFECT OF CPFB ON THE TRANSFORMATION OF BALB/c-3T3 CELLS WITH METABOLIC ACTIVATION – EXPERIMENT 4[a]

| Concentration (μg/mL) | Number of Dishes | Number of Type III Foci | Number of Type III Foci/Dish | Estimated Transformation Frequency[b] (Transformants per Cell Plated) | Calculated Transformation Rate (Transformants/Cell/Generation) as Determined by: | |
|---|---|---|---|---|---|---|
| | | | | | Mean Method[c] | $P_0$ Method[d] |
| Media Control | 12 | 0 | 0 | $2.67 \times 10^{-6}$ | $7.3 \times 10^{-8}$ | $3.02 \times 10^{-8}$ |
| DMSO (0.2%) | 11 | 0 | 0 | $3.05 \times 10^{-6}$ | $8.0 \times 10^{-8}$ | $3.30 \times 10^{-8}$ |
| B(a)P 0.25 | 12 | 13 | 1.08 | $8.00 \times 10^{-5}$ | $2.8 \times 10^{-7}$ | $6.21 \times 10^{-7}$ |
| 0.12 | 11 | 8 | 0.73 | $3.40 \times 10^{-5}$ | $2.3 \times 10^{-7}$ | $3.51 \times 10^{-7}$ |
| CPFB 1000 | 11 | 12 | 1.09 | $5.87 \times 10^{-5}$ | $2.9 \times 10^{-7}$ | $8.61 \times 10^{-7}$ |
| 500 | 12 | 7 | 0.58 | $2.38 \times 10^{-5}$ | $1.9 \times 10^{-7}$ | $3.03 \times 10^{-7}$ |
| 250 | 12 | 6 | 0.50 | $1.74 \times 10^{-5}$ | $1.7 \times 10^{-7}$ | $2.40 \times 10^{-7}$ |
| 125 | 12 | 4 | 0.33 | $1.14 \times 10^{-5}$ | $1.4 \times 10^{-7}$ | $1.41 \times 10^{-7}$ |
| 62.5 | 12 | 0 | 0 | $2.88 \times 10^{-6}$ | $7.3 \times 10^{-8}$ | $3.02 \times 10^{-8}$ |

[a] Experiment was performed on 60-mm plastic dishes.
[b] Transformation frequencies correlated for relative survival and calculated as described by Reznikoff et al. (1973).
[c] Transformation rate calculated by the mean method according to Capizzi and Jameson (1973).
[d] Transformation rate calculated by the $P_0$ method using the Luria-Delbruck fluctuation analysis (1943).

CPFB did not induce significant numbers of mutations in the HGPRT locus in CHO cells. Although the response at 500 μg CPFB was four to five times greater than the background (Tables 8.2-3 and 8.2-4), this is not sufficient to be considered a positive response; five- to six-fold increases are normally required before a result is considered positive, and at least two concentrations must show this response. The CHO/HGPRT Assay measures the ability of a test chemical to induce forward mutations at the HGPRT enzyme locus of CHO cells. Mutants at the HGPRT locus are unable to convert purine analogues, such as 6-TG, to the phosphorylated metabolites and will therefore survive in medium containing 6-TG, whereas the nonmutant cells incorporate the phosphorylated 6-TG into the DNA and cease to replicate. The assay was performed both with and without exogenous metabolic activation (Aroclor 1254-induced rat liver microsomal-S9 fraction) to permit detection of direct-acting chemicals as well as chemicals that may require activation, respectively. The results presented here for the HGPRT/CHO Mutation Assay and the solubility experiments suggest that the previous observations (Tu et al., 1986) of possible mutagenic activity with CPFB at concentrations of 50 and 250 μg/mL using DMSO as the solvent were perhaps due to the interaction of CPFB, DMSO, and the plastic dishes used for treatment.

Data from the CHO SCE Assays strongly suggest that CPFB may be genotoxic to mammalian cells. Sister chromatid exchange results from breaking and exchanging DNA from sister chromatids within the same chromosome. This assay is very sensitive and detects damage that is likely not seen in other assays. At the highest concentration of CPFB (1000 μg/mL) in two independent assays, one with and one without metabolic activation, highly significant ($p < 0.005$) increases in the mean number of SCEs per chromosome (compared to controls) were observed. These high mean values were comparable to those of the positive control compound, DMBA. In the other two assays, marginally significant ($0.005 < p < 0.05$) increases were seen only at the top two CPFB concentrations. Another indication that CPFB is positive is the trend toward higher mean SCE values with higher CPFB concentrations. This positive trend is usually a good indication of a genotoxic chemical, and the fact that it was seen in all four assays serves to strengthen the interpretation. However, such high concentrations may not be realistic in whole animals.

CPFB induced marginally significant ($0.001 < p < 0.01$) increases in CHO CAbs (excluding gaps) in all four assays, both with and without metabolic activation. These marginally significant results were four- to 11-fold less than the values obtained by exposing cells to 1.0 μg DMBA/mL (the highest dose of the positive control compound). However, a positive trend of increasing CAbs with increasing exposure concentrations suggests that some effect is present, but that it may be marginal. The ability of a given chemical to induce CAbs can be considered strongly suggestive of its ability to produce DNA lesions that could lead to serious chronic health effects.

In the Primary Rat Hepatocyte UDS Assay, CPFB did not produce significant increases in the repair of DNA damage. Among the techniques for monitoring DNA damage, DNA repair is of particular value because it is a specific response of covalent binding to DNA and is not mimicked by any other toxic effect to the cell. For the measurement of DNA repair, primary hepatocytes are advantageous in that they possess a substantial intrinsic capacity for xenobiotic transformation and, because they are nonproliferative in primary culture, inhibition of replicative DNA synthesis is not required to measure DNA repair. Previous results of UDS assays on 73 carcinogenic and 13 noncarcinogenic compounds showed that 96% of known carcinogens were positive (three carcinogens were false negatives [aniline, carbon tetrachloride, and diphenylnitrosamine]) and all 13 noncarcinogens were negative (Williams, 1981).

CPFB exposure increased the transformation frequency of BALB/c-3T3 cells. The BALB/c-3T3 transformation assay permits the evaluation of the cancer-causing properties of test chemicals. The phenotypic appearance of morphologically altered cells following exposure correlates with the subsequent ability of these cells to produce tumors in syngeneic hosts. In the two assays without metabolic activation, the transformation frequencies were lower than the positive control compounds (B[a]P). However, with metabolic activation present, the transformation frequencies induced by the highest CPFB concentration exceeded those of the positive control compound at the highest concentration. In all four transformation assays a dose-dependent increase in the transformation frequency was observed, strongly suggesting the ability of CPFB to transform cells.

Table 8.2-21 is a summary of all data. These results indicate that CPFB may have significant genotoxic activity in the SCE and CAb tests, especially at the highest concentration. In the BALB/c assay, the CPFB gave weakly positive results without metabolic activation, although with metabolic activation positive transformation rates and frequencies were observed at higher CPFB concentrations. Positive results were observed in these assays at concentrations higher than those previously tested (concentrations of up to 300 µg/mL) (Tu et al., 1986). CPFB did not show genotoxic activity in the Ames Assay, the CHO Mutation Assay, or the Primary Rat Hepatocyte Assay. These assays detect point mutations and DNA damage repair and cover the major forms of DNA damage attributable to genotoxic chemicals. In some tests, data from the 1000 µg/mL and sometimes the 500 µg/mL concentration groups suggested significant results. These positive findings indicate probable genotoxicity at very high doses; however, artifacts at such high concentrations would have to be considered in any interpretation. For instance, high osmolality and pH can induce SCEs in human lymphocytes (Morgan and Crossen, 1981; Littlefield et al., 1981). It is unlikely that such high concentrations would ever be encountered in the human situation.

## TABLE 8.2-21. SUMMARY OF RESULTS

| Assay | -S9[a] Test[c] | | +S9[b] Test[c] | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| **Gene Mutation Assays** | | | | |
| Salmonella Mutagenicity Assay | - | -- | - | - |
| Mammalian Cell Mutation Assay | - | - | - | - |
| **Mammalian Cell Cytogenetic Assays** | | | | |
| Sister Chromatid Exchange Assay | + w | + | + | + w |
| Chromosome Aberration Assay | + w | + w | + w | + w |
| **Primary Rat Hepatocyte UDS Assay** | - | - | | |
| **BALB/c-3T3 Transformation Assay** | + w | + w | + | + |

[a] Without metabolic activation
[b] With metabolic activation
[c] + = positive
+w = weakly positive
- = negative

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## SECTION 9

## ARMY CHEMICAL DEFENSE TOXICOLOGY

### 9.1    THE ACUTE TOXICITY OF o,o'-DIETHYLMETHYLPHOSPHONITE HYDROLYSIS PRODUCTS: ETHYLMETHYLPHOSPHONATE AND ETHANOL

E. Kimmel, D. Pollard, J. Young, and R. Carpenter

### INTRODUCTION

The determination of the acute inhalation toxicity of o,o'-diethylmethylphosphonite (TR) is part of a battery of investigations undertaken to evaluate the acute toxicity of several compounds associated with the manufacture and decomposition of o-ethyl-o-(diisopropylaminoethyl) methylphosphonite (QL). The acute oral, dermal, and inhalation toxicity of triethylphosphite (TEP), diisopropylaminoethanol (KB), and bis(diisopropylaminoethyl)methylphosphonite (LT), as well as the acute dermal and oral toxicity of TR, has been evaluated and reported (Vernot et al.,1984; Kinkead et al.,1987).

Evaluating the acute inhalation toxicity of TR is complicated by the chemical instability of this compound in air. A series of experiments conducted in this laboratory have demonstrated that TR is unstable in the presence of water vapor because it rapidly hydrolyzes into ethanol and ethylmethylphosphonate (YL) (to 99.8% completion in 4.5 min at RH = 50% – vida infra). TR also oxidizes to form diethylmethylphosphonate (TRO) but at a much slower rate. At relative humidities (RH) encountered in all but the most arid (below 20%) of climates, the hydrolysis of TR to YL and ethanol is the principal degradation pathway, proceeding at rates that essentially preclude the formation of TRO. Furthermore, in air the chemical degradation of TR to YL is accompanied by a phase transition from the vapor state to aerosol. This chemical and physical instability posed numerous problems requiring resolution prior to undertaking an acute inhalation toxicity study. A suitable generation system has been developed for conducting exposures to chemically stable atmospheres over a broad range of concentrations, and analytical methods have been developed to adequately characterize these atmospheres.

### METHODS AND RESULTS

The instability of TR in air is caused by its spontaneous reactivity with both water vapor and oxygen, forming either YL and ethanol or TRO and numerous other degradation products (Figure 9.1-1). In order to select the most chemically stable atmosphere for inhalation studies that was toxicologically relev... and reproducible over a broad spectrum of concentrations, it was necessary to examine the kinetics of the hydrolysis and oxidation reactions. Earlier, Pollard (1986 – unpublished) conducted a series of investigations to determine the different rates at which TR hydrolysis occurred as a function of the moisture content in the air. Known quantities of 98.8% pure TR were allowed to react with air at known RH in glass sampling bottles for specified periods of time. Gas samples then were analyzed for TR, YL, ethanol, and TRO content via gas chromatography

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(GC) using a flame ionization detector (FID). The hydrolysis of TR at various RH followed first order reaction kinetics, with the rate of reaction increasing rapidly as a function of increasing RH (Figure 9.1-2). Reaction rate constants at three RH are given in Table 9.1-1.
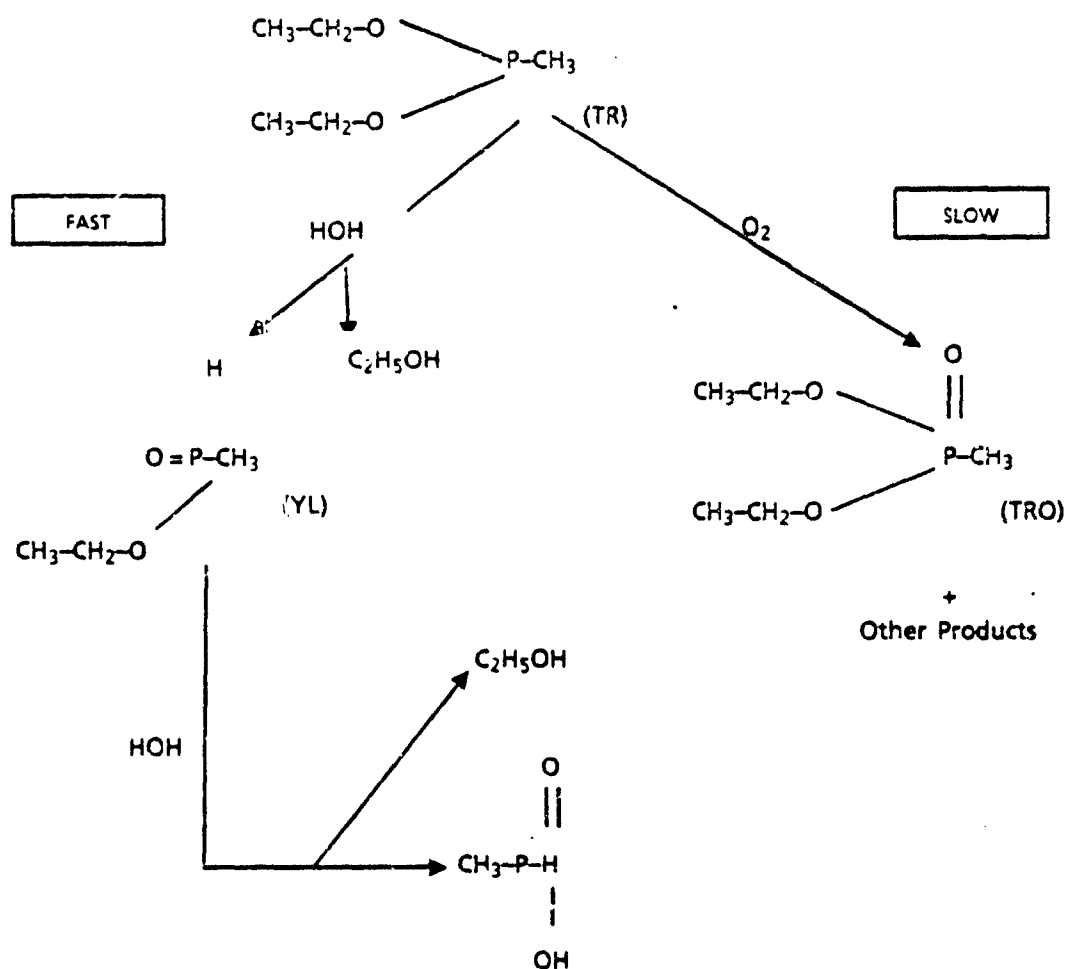


Figure 9.1-1. Spontaneous Reactions of TR in Air.

Thus, at 50% RH, the half-life of TR is approximately 0.5 min. Consequently, after 4.5 min, 99.8% of TR had hydrolyzed to YL and ethanol, which are relatively stable intermediates. The results of a series of experiments to develop a stable vapor generator for instrument calibration (described below) were used to estimate the oxidation reaction rate. TR was volatilized from a diffusion oven for up to five days with dry tank air passed through a dessicant. Periodic analysis demonstrated that the formation of TRO under these conditions was relatively slow, with a reaction half-life on the order of six days. Furthermore, the formation of YL was negligible. These data indicate that at the RH normally encountered in inhalation chambers containing animals (~50%), TR will rapidly form YL and ethanol; however, formation of TRO will not be a factor, especially in a dynamic exposure system where the residence time of air in the chamber is a matter of minutes.
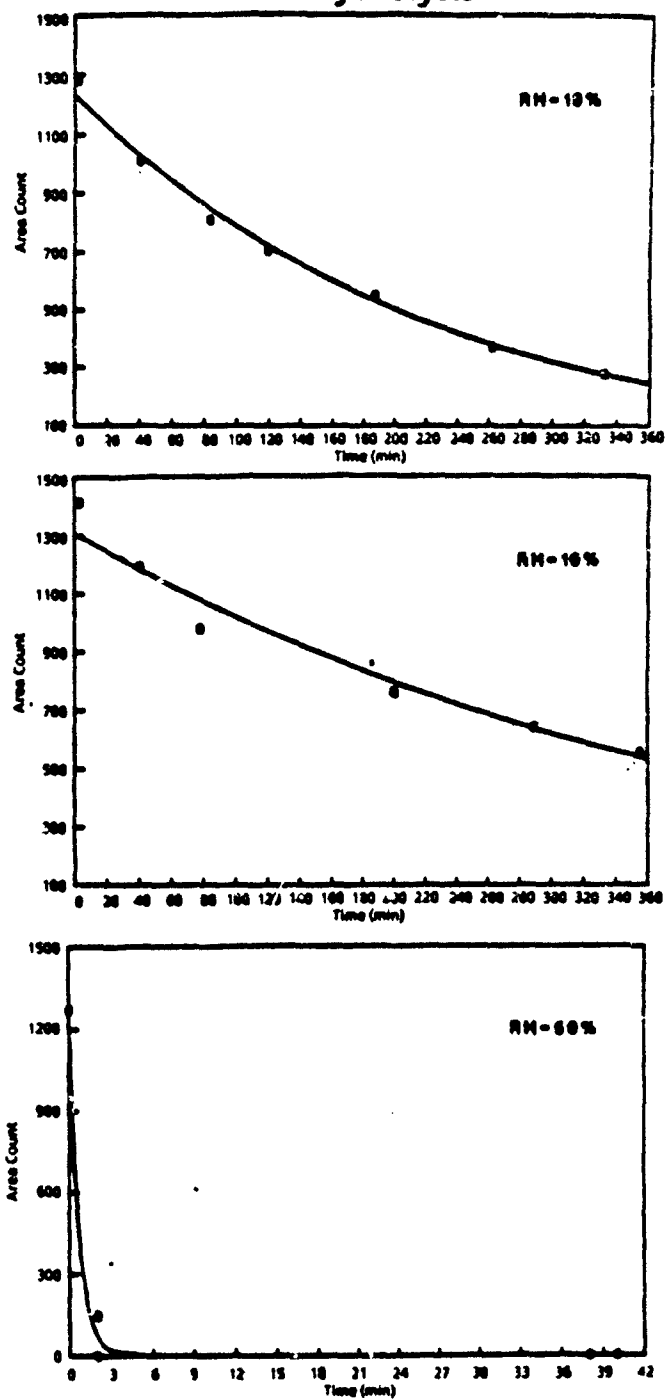
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# TR Hydrolysis



Figure 9.1-2. Kinetics of TR Hydrolysis in Air at Various RH.

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## TABLE 9.1-1. TR HYDROLYSIS REACTION RATES

| RH (%) | Rate Constant (min⁻¹) |
|--------|----------------------|
| 10 | 0.00251 |
| 19 | 0.00450 |
| 50 | 1.45410 |

To examine the reaction of TR in a dynamic flow system, a prototype generator was developed (Figure 9.1-3). Fresh samples of TR (98% pure – see Figure 9.1-4) were placed in a 100-mL round bottom flask. Clean dry air was humidified in a bubbler and flowed through the flask into a 1-L aging flask. Airflow through the system was 0.5 L min⁻¹. Assuming perfect mixing in the 1-L flask, the residence half-time in the flask was 1.4 min. The system was operated for approximately 6 h, and a liquid condensate formed n the aging flask. Analysis of the generator pot material after generation indicated that all of the TR had undergone hydrolysis and some oxidation (Figure 9.1-4). However, the condensate in the aging chamber was nearly pure YL and ethanol. Thus, in a dynamic system (flowing air), as well as in a static system, the hydrolysis products YL and ethanol are chemical constituents to which animals will be exposed under normal conditions (i.e., where RH >40% and toxicant residence time is at least on the order of minutes).
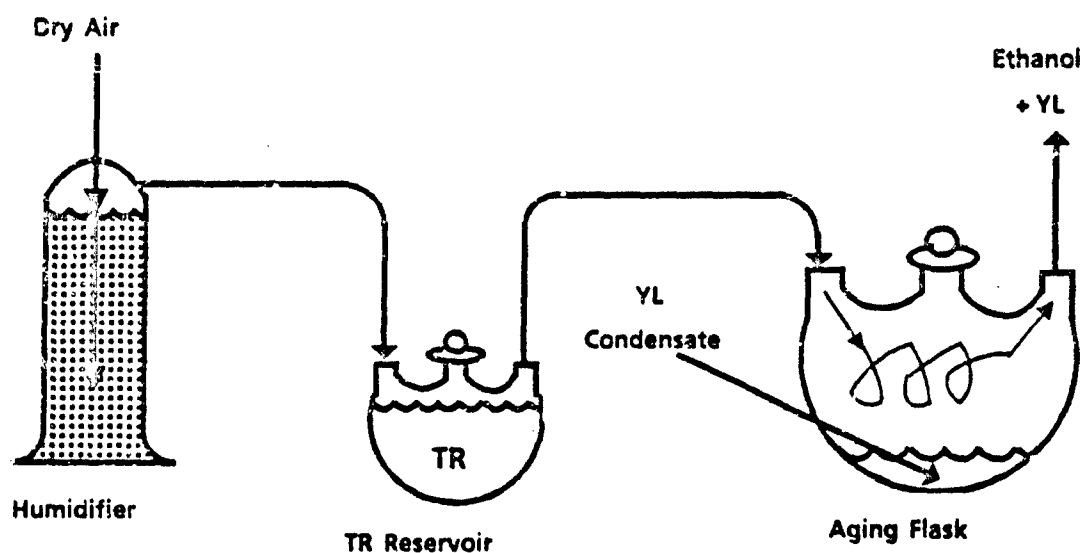


Figure 9.1-3. Schematic of a Prototype TR Vapor/Aerosol Generator, Using Moist Air for Vaporization.
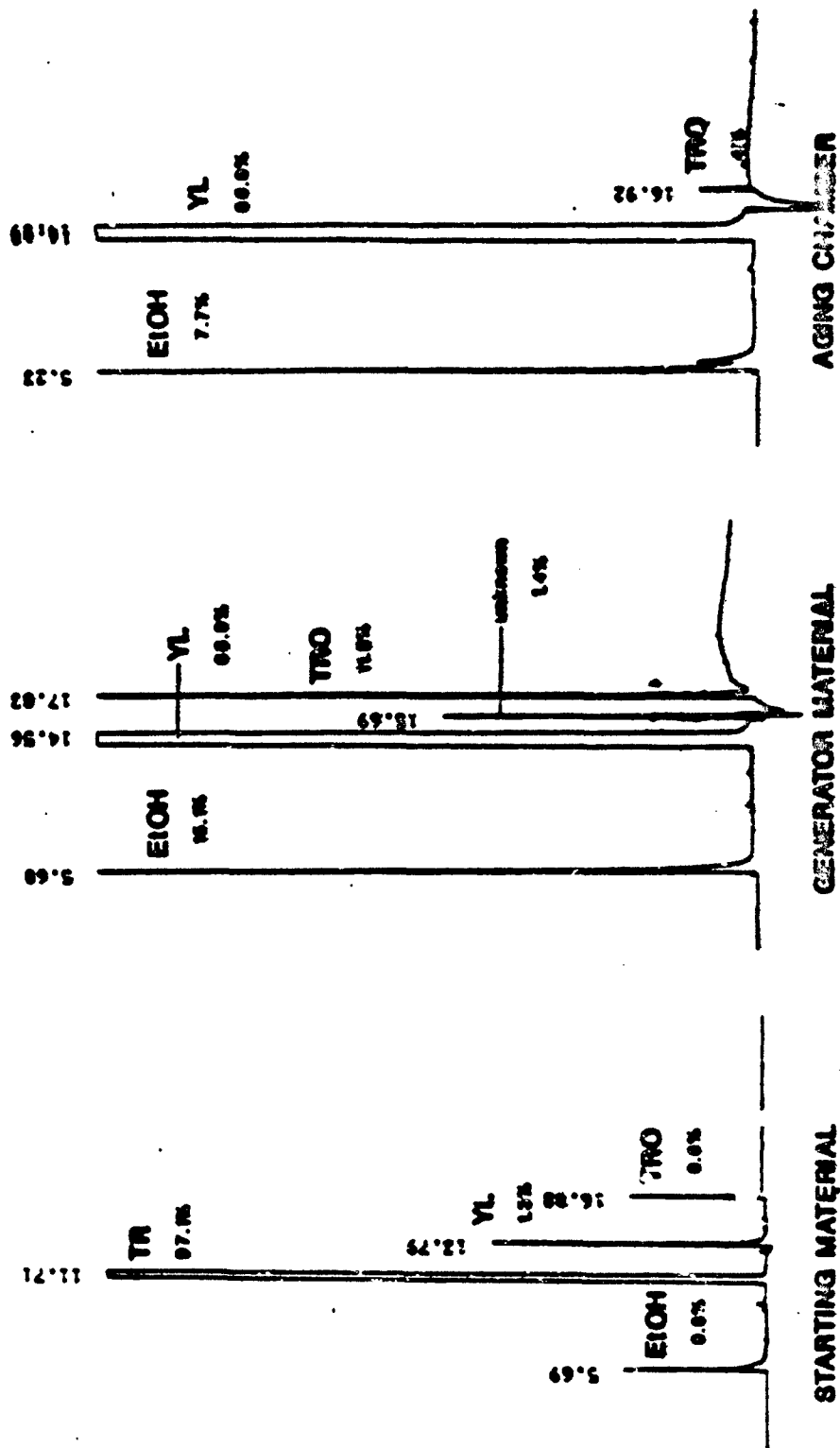
Figure 9.1-4. Chromatograms of TR and TR Degradation Products in the Prototype Generation System. Composition of the starting material, generator residue after four h and condensate in the aging flask.

A reliable, stable inhalation exposure system requires that the vapor/aerosol source produce a constant mass flow of the toxicant. The experimental data provided above demonstrate that the parent material in the prototype generator decomposes over time when air is used to vaporize the TR. To minimize both oxidation and hydrolysis of material in the generator solution, an acute inhalation system was designed that uses dry $N_2$ instead of air to vaporize the TR. A schematic of this system is shown in Figure 9.1-5. The TR bearing $N_2$ flow from the generator (bubbler) is approximately 5% of the humid air flow and is mixed with this airflow with no appreciable diminution of RH (~55%) or oxygen content. The $N_2$ and humid air are mixed thoroughly using counter current flow mechanisms, and the TR-laden $N_2$ is injected directly into $H_2O$ mist to ensure contact of the TR vapor with the humid air. This mixture is then transported through an aging chamber where the residence time of the atmosphere is 20 min; this process ensures complete hydrolysis of the TR. Twenty minutes corresponds to 40 TR half-lives; thus, theoretically, only $2.3 \times 10^{-11}\%$ of the parent TR remains unhydrolyzed. The atmosphere is then conveyed into the inhalation chamber housing the animals under study. In this manner, the exposure chamber atmosphere chemical composition and concentration remain stable throughout the exposure.
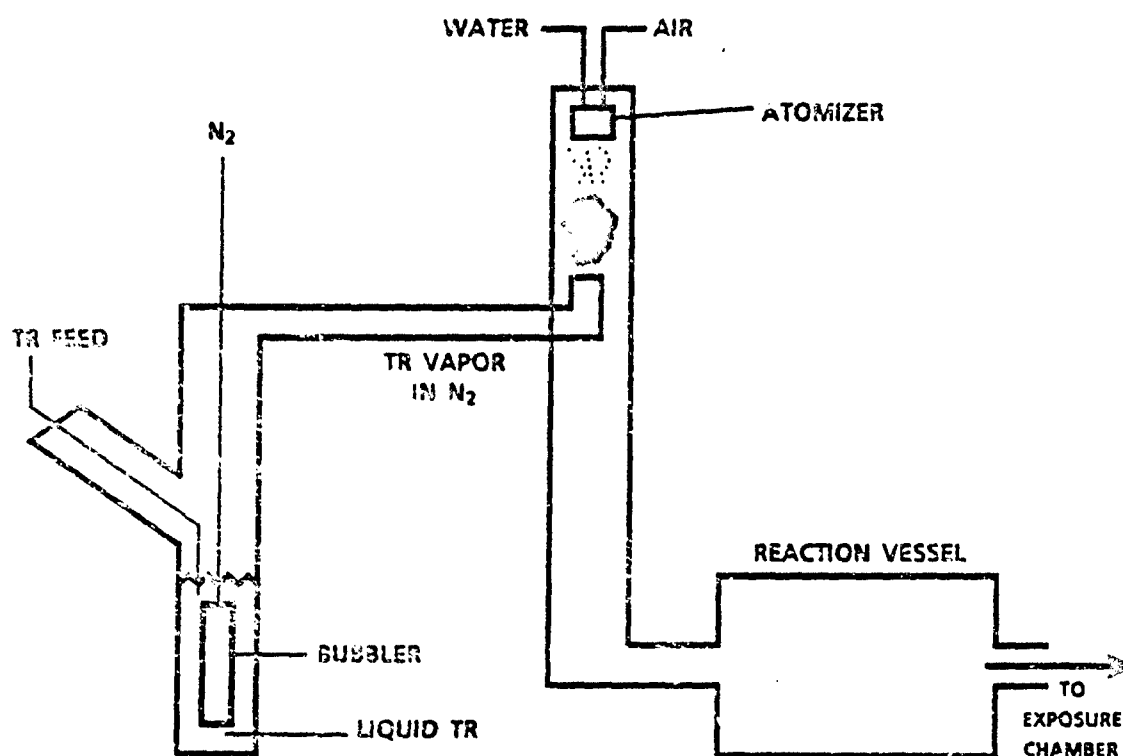


Figure 9.1-5. Schematic of the Exposure System TR/YL Generator, Using Dry $N_2$ for Vaporization.

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An experiment was conducted to examine TR atmospheric chemistry in the full-scale system described above. In this system, one 690-L THRU exposure chamber serves as the aging chamber prior to delivery of the toxicant atmosphere to a second 690-L THRU exposure chamber housing the experimental animals (no animals were used). The system was operated for 4 h at a flow of 150 L min$^{-1}$ (13 chamber volumes/h), 2 in. $H_2O$ subambient pressure, 22°C and 56% RH. Chamber phosphorus content was monitored to determine TR equivalents (*vida infra*), and exposure chamber concentration ranged from 0.087 mg/L to 0.090 mg TR/L equivalents. Chromatograms of the starting material, material in the generator after the run, and of the chamber atmosphere (Figure 9.1-6) show that the exposure atmosphere was all YL and ethanol. There was no difference in the chemical composition of the TR in the generator before and after the run (Figure 9.1-6).

During the inhalation exposures, chamber concentration of TR hydrolysis products will be documented using a flame photometric method specific for phosphorus (Columbia Southern Instruments - model PA 260 - HYFED). The detector burns the sample in a hydrogen/air flame and uses wavelength-specific filters and a photomultiplier to determine the intensity of the phosphorus emission. In this manner, moles of phosphorus per unit volume of atmosphere can be determined and from this the molar equivalents of parent TR can be calculated. The HYFED detector was calibrated with several organophosphate compounds. Using dimethylmethylphosphonate (DMMP) the HYFED was found to have a linear response up to 10 ppm phosphorus (Figure 9.1-7). At levels higher than 10 ppm, the analyzer responds in plateaus.

Several approaches were used to calibrate the HYFED detector with TR and YL. Initially, Teflon® standard bags were prepared with known volumes of dry air and TR. The results of these measurements were inconsistent. Chromatography of the bag's contents indicated that TR was oxidizing, and presumably TRO was absorbing to the bag walls. Therefore, the experiments were repeated using dry $N_2$ to prevent oxidation. Although there was no evidence of TR oxidation, it was apparent that TR also absorbed on the bag walls, hence the concentration in the bag could not be determined accurately.

Since plastic surfaces led to absorption of TR, an all-glass diffusion tube system was developed for calibration of the detector. In this system, small glass tubes were filled with TR. These tubes had an orifice in the top through which TR could diffuse. The tubes were placed in an oven and warmed slightly to control the diffusion rate. The amount of TR evaporated was determined by the weight loss of the tubes. During this time, dry air was passed through the diffusion oven and transported into the HYFED detector. Again, the results were inconsistent, and chromatography indicated that the TR was oxidizing and hydrolyzing due to back diffusion of water vapor into the oven. Therefore, the chemical composition of the material in the tubes was changing with time, and the assumption that tube weight difference represented TR vaporization was not valid. Nitrogen can not be used in
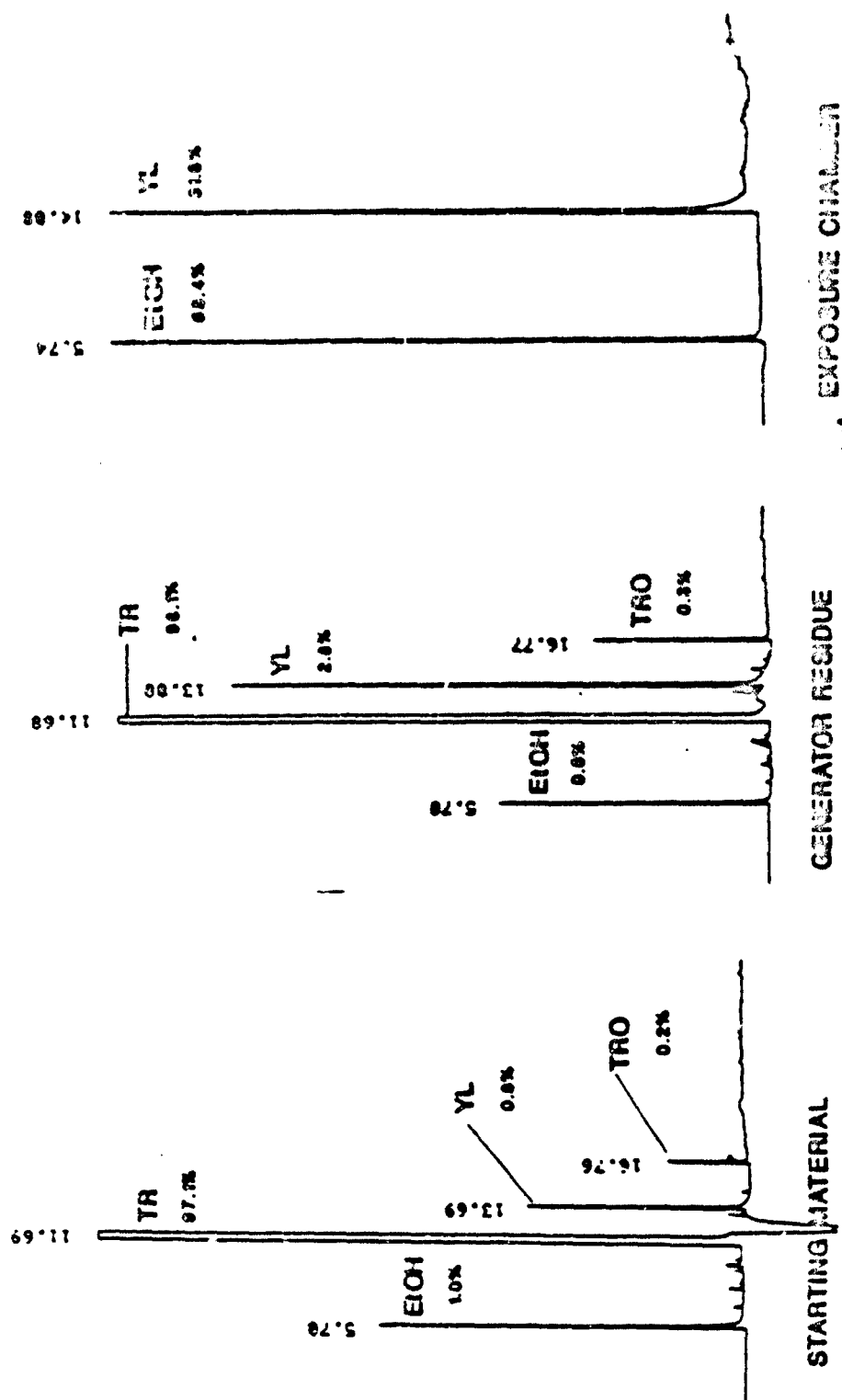
Figure 9.1-6. Chromatograms of TR and TR degradation products in the exposure system generator. Composition of starting material, generator residue after four h and exposure atmosphere.
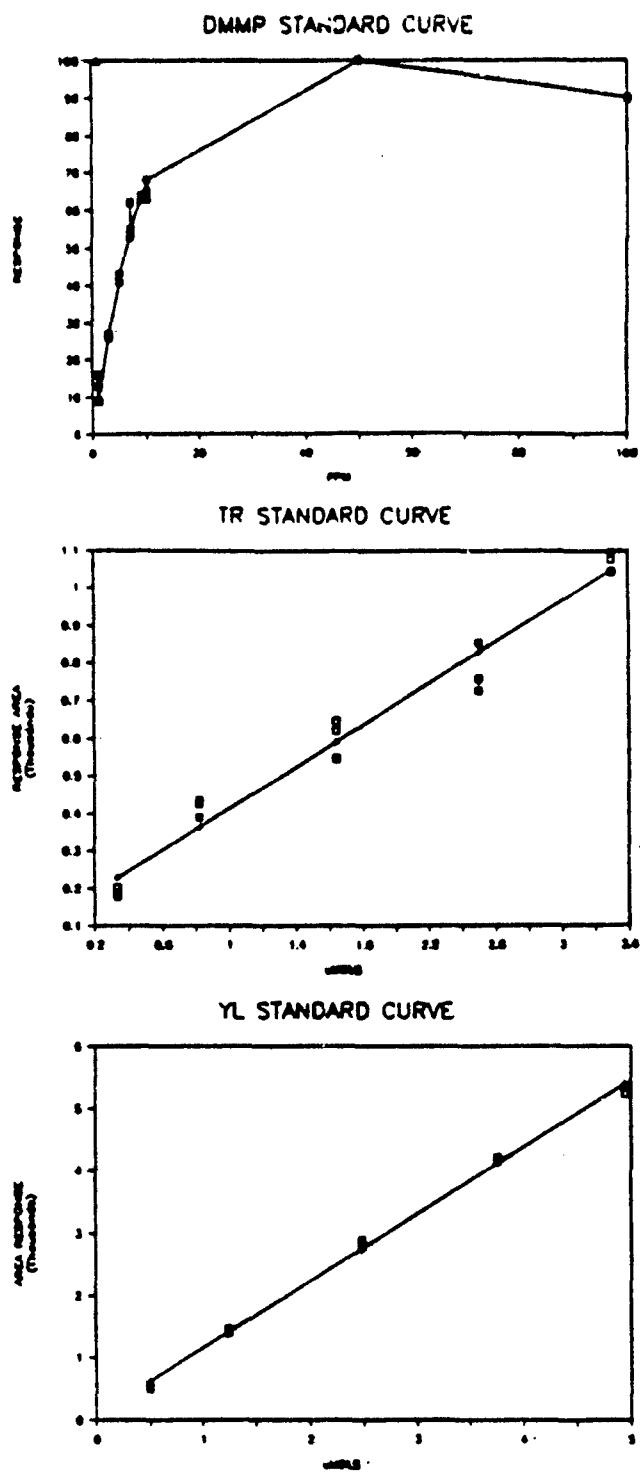
Figure 9.1-7. Calibration Curves for the HYFED Detector with Selected Organophosphates.

this system because the HYFED requires oxygen in the sample stream for combustion. An alternative method for calibrating and sampling had to be developed. A special stainless-steel fitting was developed that allowed air to pass through a stainless-steel frit. Access to the downstream side of the frit was through a septum similar to that on a GC. Known volumes of TR were injected onto the frit using a microliter syringe. The HYFED detector responded instantaneously to the TR, producing a peak similar to that of a chromatograph. In effect, with this method of operation, the HYFED was a GC without a column. The area under the response peak was proportional to the amount of TR injected, and the response of the detector was linear (Figure 9.1-7). With this mode of operation, the HYFED can not be used as a real time, continuous monitor of chamber concentration; however, the analytical range of the instrument was expanded to well over the previous 10 ppm limitation. Pure YL was obtained by controlled hydrolysis of TR and vacuum distillation. The HYFED detector was calibrated with YL (Figure 9.1-7). Again, the response of the detector was linear. The calibration system was adapted for use as the exposure chamber concentration monitoring system.

## DISCUSSION

At present, additional test runs of the exposure and sampling system at higher concentrations are required to determine the maximum exposure concentration of TR equivalents that can be measured. Conduct of the inhalation exposures is pending final development and approval of the experimental protocol.

## REFERENCES

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Vernot, E.H., J.D. MacEwen, E.R. Kinkead, J.R. Horton, J.D. Collins, W.J. Bashe, C.C. Haun, A.K. Roychowdhury, R.A. Salomon, and E.J. Olajos. 1984. Studies on the acute toxicity of o-ethyl-o'-(diisopropylaminoethyl)methylphosphonite (EDMP). Technical Report #CRDC-CR-84116, U.S. Army Armament, Munitions and Chemical Command, Aberdeen Proving Ground, MD.

## TOXICOLOGY OF NAVY MATERIALS

### 10.1 DETERMINATION OF THE ACUTE SKIN IRRITATION OF AN "ACTIVATED PEROXIDE" USING VARIED EXPOSURE REGIMENS

E. Kinkead, B. Culpepper, and S. Henry

*INTRODUCTION*

The Navy has expressed interest in a decontamination suspension for removing chemical warfare agents from equipment and possibly personnel. The material will be distributed in a dispenser containing the solid peroxide/clay ingredients and a liquid in a separate inner membrane, so that when squeezed the slurry would mix, releasing the activated peroxide. An applicator top, much like liquid shoe polish, then would be used to apply the suspension to clothing or small parts.

Hydrogen peroxide, a component of sodium carbonate peroxide, is caustic and at sufficiently high concentrations causes skin and eye irritation. Hydrogen peroxide is the most toxic component of the decontaminant mixture. The expected primary route of exposure to the mixture would be as a dermal agent in a dry or hydrated form. It was reported earlier (Kinkead *et al.*, 1987) that "activated peroxide" is too irritating for human skin contact. This determination was made as a result of skin irritation tests in which the decontaminant was in contact with the rabbits' skin for 4 h.

It is anticipated that situations may arise in which use of the decontaminant on human skin may be a necessary or preferred treatment. The Naval Medical Research Institute/Toxicology Detachment (NMRI/TD) has requested that further rabbit skin tests be performed using reduced time periods of skin contact, followed by a soap or detergent wash to determine at what time postexposure the "activated peroxide" can be washed from the skin without producing an irritating lesion.

*MATERIALS*

*Test Material*

The test materials supplied by the NMRI/TD consist of the following agents.

| Sample | NMRI/TD No. |
|---|---|
| DCON Mix (per bag): | 86-241-2, a-j |
|    26 g   Sodium percarbonate | |
|    39 g   Clay blend | |
| Solvent Mix (per bag) | 86-241-1, a-j |
|    0.1 %   Triton X-100 | |
|    15.0 %   Butyl carbitol | |
|    1.0 %   Didecyl dimethyl ammonium | |
|         Remainder water | |

Total Volume per bag ≈ 200 mL

The test sample, a thorough mix of 20 mL of solvent mix and 6.5 g of DCON mix, was prepared within 30 min of use for eacn of the irritation tests. Prior to testing, the pH of the slurry was determined with the intention that predictable corrosive materials (pH <2.0 or >11.5) would not be tested.

### Animals

Female New Zealand White rabbits, weighing 2 to 3 kg, were purchased from Clerco Research Farms, Cincinnati, OH. Health assessment upon arrival showed the animals to be in acceptable health. The rabbits were housed singly in stainless-steel cages with wire-mesh floors. Water and food (Purina Rabbit Chow #5320) were provided ad libitum. All rabbits were sacrificed by IV T-61 Euthanasia Solution (Taylor Pharmacal Co., Decatur, IL) overdose at the termination of the observation period.

### METHODS

#### Primary Skin Irritation Test

The rabbit skin irritation tests were performed on groups of six rabbits each (topically dosed with 0.5 mL of the test agent to a clipped patch area) per contact time period (see Table 10.1-1). Each group was used for two series of tests (the shorter time period first), one week apart, using two different sites on the rabbits' backs. In the first series of exposures, 30 min or less, the test site was not covered by a semiocclusive dressing. At the end of the exposure period, the test site was washed and thoroughly rinsed with a mild cleansing soap (Liquid Neutrogena Facial Cleansing Formula [Product # 117], Neutrogena Corp., Los Angeles, CA). In the second series of exposures, the test site was covered by a semiocclusive dressing for 30, 60, 120, or 240 min. The semiocclusive dressing consisted of a 2.5-cm square of surgical gauze two single layers thick, which was held in place with strips of Elastoplast (Elastoplast, Beirsdorf Inc., Norwalk, CT) tape. The entire area was covered with dental dam and secured with Vetrap (Vetrap, 3M Corp., Minneapolis, MN) and Elastoplast tape. The patch remained in place for the specified exposure period, at which time all wrappings and excess test material were removed. A mild cleansing soap was used to wash the "activated peroxide" from the skin.

Examination for signs of skin irritation were made at 4, 24, 48, and 72 h following application. Scoring of the irritative effects was made according to the method of Draize (1959). The total score of the four observations for all rabbits in each group was divided by 24 to yield a primary irritation rating, which was interpreted using the NIOSH skin test rating of Campbell et al. (1975; Table 10.1-2).

**TABLE 10.1-1. EXPOSURE REGIMEN FOR RABBIT SKIN IRRITATION TESTS**

| Time Period (min) | Semiocclusive Dressing | Number Rabbits | Assessment |
|---|---|---|---|
| 5 | No | 6 | (All groups) |
| 10 | No | 6 | Skin Irritation |
| 15 | No | 6 | |
| 30 | No | 6 | |
| 30 | Yes | 6[a] | |
| 60 | Yes | 6[a] | |
| 120 | Yes | 6[a] | |
| 240 | Yes | 6[a] | |

[a] Rabbit group previously tested at shorter time period.

**TABLE 10.1-2. NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH INTERPRETATION OF INTACT SKIN TEST RATINGS[a]**

| Rating | Interpretation |
|---|---|
| 0-0.9 | Nonirritant; probably safe for human skin contact |
| 1-1.9 | Mild irritant; may be safe for use, but appropriate protective measures are recommended during contact |
| 2-4.0 | Too irritating for human skin contact; avoid contact |

[a] Campbell et al., 1975

## RESULTS

Prior to rabbit-skin irritation testing, the pH of the slurry mixture was determined. The material was moderately alkaline with a pH of 10.5. Six female rabbits per test group were used to measure the primary dermal irritation of the decontamination compound on intact skin. The groups of rabbits tested on nonoccluded sites for 30 min or less showed no signs of erythema or edema at the 4-h observation period (Table 10.1-3). Erythema was evident by 24 h in each group except for the rabbits exposed for 5 min, where signs of erythema were delayed to 48 h. Edema was noted in only one rabbit, at 48 h in the group having 30-min contact with the test material.

Occlusion of the test sites for 30 min or more markedly enhanced the irritative effects of the decontaminant material (Table 10.1-4). Erythematous responses were evident by 24 h or less in most rabbits, while edema was most evident after 24 h. Necrosis was not noted in any of the rabbits at any observation period.

## TABLE 10.1-3. TOTAL SCORES[a] OF THE EVALUATION OF NONOCCLUDED RABBIT SKIN REACTIONS TO THE TEST MATERIAL

| | Time of Contact (min) | | | |
|---|---|---|---|---|
| | 5 | 10 | 15 | 20 |
| **4-h Observations** | | | | |
| Erythema | 0 | 0 | 0 | 0 |
| Edema | 0 | 0 | 0 | 0 |
| Necrosis | 0 | 0 | 0 | 0 |
| **24-h Observations** | | | | |
| Erythema | 0 | 1 | 5 | 3 |
| Edema | 0 | 0 | 0 | 0 |
| Necrosis | 0 | 0 | 0 | 0 |
| **48-h Observations** | | | | |
| Erythema | 3 | 5 | 0 | 8 |
| Edema | 0 | 0 | 0 | 1 |
| Necrosis | 0 | 0 | 0 | 0 |
| **72-h Observations** | | | | |
| Erythema | 3 | 7 | 7 | 8 |
| Edema | 0 | 0 | 0 | 0 |
| Necrosis | 0 | 0 | 0 | 0 |
| **Total (All Observations)** | 6 | 13 | 22 | 20 |
| **Primary Irritation Rating** | 0.3 | 0.5 | 0.9 | 0.8 |

[a] Total score of six rabbits per observation time.

## TABLE 10.1-4. TOTAL SCORES[a] OF THE EVALUATION OF OCCLUDED RABBIT SKIN REACTIONS TO THE TEST MATERIAL

| | Time of Contact (h) | | | |
|---|---|---|---|---|
| | 0.5 | 1.0 | 2.0 | 4.0 |
| **4-h Observations** | | | | |
| Erythema | 8 | 6 | 10 | 2 |
| Edema | 0 | 0 | 0 | 0 |
| Necrosis | 0 | 0 | 0 | 0 |
| **24-h Observations** | | | | |
| Erythema | 14 | 11 | 13 | 8 |
| Edema | 4 | 0 | 3 | 0 |
| Necrosis | 0 | 0 | 0 | 0 |
| **48-h Observations** | | | | |
| Erythema | 14 | 9 | 13 | 9 |
| Edema | 8 | 2 | 6 | 5 |
| Necrosis | 0 | 0 | 0 | 0 |
| **72-h Observations** | | | | |
| Erythema | 14 | 11 | 12 | 10 |
| Edema | 8 | 1 | 3 | 8 |
| Necrosis | 0 | 0 | 0 | 0 |
| **Total (All Observations)** | 70 | 40 | 60 | 42 |
| **Primary Irritation Rating** | 2.9 | 1.7 | 2.5 | 1.8 |

[a] Total score of six rabbits per observation time

## DISCUSSION

Time periods of 30 min or less of nonoccluded skin contact, followed by a detergent wash, resulted in primary irritation ratings which would be interpreted as "probably safe for human skin contact" using the NIOSH Skin Test Rating System (Table 10.1-2). If the decontaminant is washed from the skin within 5 to 10 min, only minor skin irritation should result. Little difference is noted between the primary irritation rating at 15 min when compared to that at 30 min.

However, it is obvious that occlusion of the skin enhances the irritative effects of the decontaminant, as indicated by the primary irritation ratings of all test groups. Maximum irritative effects occur by 30 min and do not progress if the contaminant remains in contact with the skin for longer time periods. This is, most likely, directly related to the relatively short activation period of the decontaminant mixture.

The caustic nature (pH of 10.5) of this mixture, as well as the results of this investigation and the previous irritation study (Kinkead et al., 1987) in which severe skin damage was reported, indicate that personnel working in decontamination environments should wear protective clothing and avoid contact of the mixture with the skin. However, accidental or inadvertent skin contact with the decontaminant mixture should result in only mild skin irritation if the contact site is washed thoroughly with water and detergent within 30 min.

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Draize, J.H. 1959. Dermal toxicity, appraisal of the safety of chemicals in food, drugs, and cosmetics. The Editorial Committee of the Associates of Food and Drug Officials of the United States. Austin, TX: The Federal Drug Administration.

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## TOXICOLOGY OF NAVY HYDRAULIC FLUIDS AND LUBRICANTS

### 11.1 DETERMINATION OF THE TOXICITY OF CYCLOTRIPHOSPHAZENE HYDRAULIC FLUID BY REPEATED ADMINISTRATION

E. Kinkead, B. Culpepper, S. Henry, E. Kimmel,
C. Doarn, M. Porvaznik*, and R. Kutzman

### INTRODUCTION

The Navy has developed candidate hydraulic fluids with the chemical structure of cyclotriphosphazene (CTP) cyclic esters. The candidate hydraulic fluid of current interest contains 0.1% tolyltriazole, an additive that inhibits copper corrosion. Acute toxicity studies on this hydraulic fluid have been conducted (Kinkead and Bowers, 1985; Kinkead and Bashe, 1987). These studies showed that the hydraulic fluid was nontoxic by the oral and dermal routes of administration. Eye and skin irritation and skin sensitization tests also proved negative (Kinkead and Bowers, 1985). The hydraulic fluid was not detected in blood or urine of rats following exposure by aerosol inhalation or dermal contact (Kinkead and Bashe, 1987). The additive, tolyltriazole, has a reported rat oral $LD_{50}$ of 675 mg/kg (HCR, 1972).

The Toxicology Detachment of the Naval Medical Research Institute (NMRI/TD) requested the THRU to study the effects of repeated exposure by both the dermal and inhalation routes. These studies are designed to permit the determination of a no-observed-effect level and toxic effects associated with repeated or continuous exposure to CTP over a limited time; however, they are not intended to identify those effects that have a long latency period (e.g., carcinogenicity, life shortening). The selected species and group sizes used in these studies conform with the U.S. Environmental Protection Agency's Health Effects Test Guidelines (1982).

### MATERIALS

#### Test Agent

The cyclotriphosphazene cyclic ester hydraulic fluid contains 0.1% tolyltriazole. It was supplied by NMRI/TD, Wright-Patterson Air Force Base, OH. Pertinent data on these materials are provided below.

---

Cyclotriphosphazene ester:

| | |
|---|---|
| NMRI/TD No. | 4341-1 |
| Vapor Pressure, mmHg | 0.49 @ 65°C |
| | 12.0 @ 149°C |
| Specific Gravity (g/mL) | 1.445 (water = 1.0) |

Tolyltriazole: $C_7H_7N_3$

| | |
|---|---|
| CAS No. | 29385-43-1 |
| Synonyms | Methylbenzotriazole |
| | 1, 2, 3-Triazole |
| | (methylphenyl) |

This hydraulic fluid is a mixture of parent compound isomers and contains dimers, trimers, and tetramers of cyclotriphosphazene. The estimated molecular weight of the fluid is 1000 g/mole.

## Test Agent Quality Control

A Varian 3700 gas chomatograph (GC) equipped with a flame ionization detector (FID) and a 50-m, 5% phenylmethyl silicone capillary column was used in conjunction with a Hewlett-Packard 3388 computing integrator to measure peak area and record chromatograms of the test material. Profiles were obtained of the material as received, aerosolized, and residue from the nebulizer system.

## Animals

Male and female Fischer-344 (F-344) rats, 9 to 11 weeks of age at the start of the study, were purchased from Charles River Breeding Labs, Kingston, NY. The animals were randomized upon receipt and were shown to be in good health following a two-week quarantine period. They were group housed (two to three per cage) in clear plastic cages with wood-chip bedding prior to the study. The rats were housed individually and assigned to specific exposure cage locations during the study. The exposure cages were rotated clockwise (moving one position) within the THRU inhalation chambers each exposure day. Water and feed (Purina Formulab # 5008) were available ad libitum, except during the inhalation exposure period and when the rats were fasted for 10 h prior to sacrifice. The light/dark cycle was set at 12-h intervals.

Male and female New Zealand White rabbits weighing between 2 and 3 kg were obtained from Clerco Research Farms (Cincinnati, OH) for use in the dermal studies. Quality control assessments confirmed the acceptable health of the proposed study animals. The rabbits were housed individually in wire-bottom stainless-steel cages. Water and food (Purina Rabbit Chow #5320 and Carnation Rabbit Chow) were available ad libitum, and the rabbits were maintained on a 12-h light/dark cycle.

## METHODS

### Inhalation Toxicity

Exposure atmospheres were generated as aerosols using either a one-, three-, or six-jet Collison (BGI, Inc., Waltham, MA) compressed air nebulizer. The number of jets per nebulizer was determined by the desired aerosol concentration. Aerosol concentration within the exposure chamber was determined by gravimetric analysis of aerosol collected on glass-fiber filter media. The size distribution of the aerosols was determined using a Lovelace Multijet Cascade Impactor (Intox Products, Albuquerque,NM). A GC chemical analysis of additional impactor samples was performed to determine stability and overall composition of the aerosol and the material used to generate the aerosol.

Ten male and ten female F-344 rats, age 9 to 11 weeks, were placed in each of four 690-L Toxic Hazards Research Unit (THRU) inhalation chambers, which are a modification (Kimmel & Carpenter, 1987) of the Hinners (1968) exposure chamber. The animals were exposed for 6 h/day, 5 days/week for 3 weeks (15 exposures over a 21-day test period), to air only, 0.25, 0.50, or 1.00 mg CTP/L, respectively. Airflow through the chambers was maintained at 12 to 15 chamber volumes/h.

### Dermal Toxicity

Ten male and ten female New Zealand White rabbits were used for each of four dose groups as follows: 0.25, 0.50, 1.00 g CTP/kg, and 1.00 g mineral oil/kg (control group). Animals were dosed on weekdays only, for three weeks (15 doses over a 21-day test period), and the test agent was kept in contact with the skin for 6 h/day. The dose volumes were calculated from individual body weights and adjusted weekly.

Hair was clipped from the rabbits' backs prior to the first dose and twice a week thereafter throughout the study. The dose was applied to the bare skin and covered by four-ply gauze squares. The gauze was covered by a clear plastic wrap (Glad Cling Wrap, First Brands Corp., Danbury, CT) surrounding the entire midsection of the body, which, in turn, was covered by an elastic bandage (Vetrap, 3/M Corp., Minneapolis, MN). After 6 h, the tape, plastic wrap, and gauze were removed, and residual material was wiped from the skin.

## EXPERIMENTAL EVALUATIONS

### Inhalation Toxicity

Records were maintained for body weights (day 0, 7, 14, and 21), signs of toxicity, and mortality. At sacrifice, gross pathology was performed, and tissues were harvested for histopathologic examination (Table 11.1-1). In addition, blood was drawn for hematology

(Table 11.1-2) and clinical chemistry (Table 11.1-3) assays. Wet tissue weights were determined on adrenals, brain, heart, kidneys, liver, lungs, ovaries (females), spleen, testes (males), and thymus.

### TABLE 11.1-1. TISSUES HARVESTED FOR HISTOPATHOLOGIC EXAMINATION

| | |
|---|---|
| Gross lesions | Thymus |
| Thyroid/parathyroid | Brain |
| Lungs | Kidneys |
| Trachea | Adrenals |
| Hea:: | Pancreas |
| Liver | Gonads |
| Spleen | Nasal turbinates (three sections) |
| Duodenum | Uterus (females) |
| Jejunum | Esophagus |
| Ileum | Stomach |
| Urinary bladder | Colon |
| Mandibular lymph nodes | Rectum |
| Mesenteric lymph nodes | Sternum |
| Eye | Sciatic nerve |
| Preputial glands | Skeletal muscle |
| Pituitary glands | |

### TABLE 11.1-2. HEMATOLOGY ASSAYS PERFORMED ON WHOLE BLOOD

Hematocrit

Hemoglobin

Red blood cell count

Total and differential leucocyte count

### TABLE 11.1-3. SERUM CHEMISTRY PARAMETERS ASSESSED

| | |
|---|---|
| Creatinine | Alkaline phosph.ate |
| Chloride | Blood urea nitrogen |
| Calcium | Serum glutamic-pyruvic transaminase |
| Phosphorus | Serum glutamic-oxalacetic transaminase |

*Dermal Toxicity*

Body weights were measured on days 0 (just prior to the first dose), 7, 14, and 21. The animals were observed daily, and any signs of toxicity were recorded. Test and control groups were sacrificed on the day following the 15th dose. On the day of sacrifice, gross pathology was performed and tissues (Table 11.1-4) harvested for histopathologic examination. In addition, blood was drawn one day prior to the first dose, and again at sacrifice for hematology (Table 11.1-2) and clinical chemistry (Table 11.1-3) assays. Wet tissue weights were determined at sacrifice on adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, testes, and thymus.

### TABLE 11.1-4. TISSUES HARVESTED FOR HISTOPATHOLOGIC EXAMINATION

| | |
|---|---|
| Gross lesions | Thymus |
| Normal and treated skin | Brain |
| Lungs | Kidneys |
| Trachea | Adrenals |
| Heart | Pancreas |
| Liver | Gonads |
| Spleen | Uterus (females) |
| Duodenum | Esophagus |
| Jejunum | Stomach |
| Ileum | Cecum |
| Urinary bladder | Colon |
| Madibular lymph nodes | Rectum |
| Mesenteric lymph nodes | Sternum |
| Gallbladder | Sciatic nerve |
| Pituitary | Thyroid/parathyroid |
| Skeletal muscle | Eye |

## STATISTICAL ANALYSIS

Body weight mean ± standard error of the mean (S.E.M.) was calculated according to Dixon (1985). Comparison of body weights was performed using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A two-factorial analysis of variance with multivariate comparisons was used to analyze the hematology, clinical chemistry, and organ weight data. The histopathology data will be analyzed using one of the following nonparametric tests: Fisher's Exact Test or, if not valid, Yates' Corrected Chi-square (Zar, 1974). A probability of 0.05 or less inferred a significant change from controls.

## RESULTS

### Chamber Analysis

The overall composition of the test material prior to aerosolization was compared to both the generator residues and the aerosol samples collected on filters. The composition of the generator material did not change during the 6-h study, and the generated aerosol had the same overall mixture of components as the starting material.

The nominal desired concentrations of 0.25, 0.5, and 1.0 mg/L were maintained during the three-week exposure period. Mean daily concentrations for each exposure chamber, along with the overall mean concentrations, are provided in Table 11.1-5. All chamber daily mean concentrations were maintained within 10% of desired, except for the 13th exposure day where the mean daily concentration of the low concentration exposure atmosphere was 84% of nominal. Because female rat exposures were started one day after the male rats, the calendar days for the complete study numbered 16; however, each group received only 15 exposures.

A summary of aerosol particle size measured in the animal exposures is listed in Table 11.1-6. Examination of particle size information indicates that the aerosols produced in all exposure chambers were of respirable size.

Animal data collected during the study are currently being analyzed and will be included in a future final report.

**TABLE 11.1-5. MEASURED DAILY MEAN[a] CONCENTRATIONS OF CYCLOTRIPHOSPHAZENE IN ANIMAL EXPOSURE CHAMBERS**

| Study Day | Target Concentrations | | |
|---|---|---|---|
| | 0.25 mg/L | 0.50 mg/L | 1.00 mg/L |
| 1[b] | 0.25 ± 0.02 | 0.49 ± 0.03 | 1.02 ± 0.09 |
| 2 | 0.24 ± 0.03 | 0.50 ± 0.03 | 1.01 ± 0.06 |
| 3 | 0.25 ± 0.04 | 0.51 ± 0.03 | 1.01 ± 0.04 |
| 4 | 0.25 ± 0.03 | 0.51 ± 0.02 | 0.96 ± 0.03 |
| 5 | 0.26 ± 0.03 | 0.50 ± 0.01 | 0.98 ± 0.03 |
| 6 | 0.25 ± 0.03 | 0.49 ± 0.02 | 0.98 ± 0.12 |
| 7 | 0.23 ± 0.04 | 0.52 ± 0.05 | 0.98 ± 0.05 |
| 8 | 0.24 ± 0.02 | 0.52 ± 0.01 | 1.00 ± 0.02 |
| 9 | 0.25 ± 0.02 | 0.51 ± 0.01 | 1.01 ± 0.05 |

(continued)

TABLE 11.1-5. (Continued)

| Study Day | Target Concentrations | | |
|---|---|---|---|
| | 0.25 mg/L | 0.50 mg/L | 1.00 mg/L |
| 10 | 0.26 ± 0.01 | 0.51 ± 0.03 | 0.93 ± 0.05 |
| 11 | 0.25 ± 0.02 | 0.50 ± 0.03 | 1.00 ± 0.15 |
| 12 | 0.23 ± 0.01 | 0.51 ± 0.03 | 0.99 ± 0.06 |
| 13 | 0.21 ± 0.06 | 0.50 ± 0.03 | 0.99 ± 0.11 |
| 14 | 0.24 ± 0.05 | 0.51 ± 0.04 | 1.01 ± 0.06 |
| 15 | 0.25 ± 0.02 | 0.52 ± 0.03 | 0.98 ± 0.08 |
| 16c | 0.25 ± 0.01 | 0.51 ± 0.02 | 1.00 ± 0.04 |
| Overall Mean | 0.24 ± 0.01 | 0.51 ± 0.01 | 0.99 ± 0.02 |

a Time weighted average ± SD.

b Male rats only.

c Female rats only.

### TABLE 11.1-6. PARTICLE SIZE OF AEROSOLS IN CYCLOTRIPHOSPAZENE INHALATION EXPOSURES

| Target Conc. (mg/L) | MMAD (μm)[a] | GSD (range)[b] |
|---|---|---|
| 0.25 | 2.10 ± 0.11 | 1.67 - 2.33 |
| 0.50 | 1.96 ± 0.01 | 1.63 - 2.18 |
| 1.00 | 2.22 ± 0.01 | 1.80 - 2.32 |

a Mass Median Aerodynamic Diameter, ± S.E.M. (n = 30).

b Geometric SD.

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## 11.2   THE DETERMINATION OF THE ACUTE AND SUBCHRONIC ORAL TOXICITY OF HALOCARBON OIL, SERIES 27-S

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### INTRODUCTION

In response to a request initiated by the Naval Sea Systems Command, the Naval Medical Research Institute, Toxicology Division (NMRI/TD) requested that the Toxic Hazards Research Unit (THRU) investigate the acute and subchronic oral toxicity of Halocarbon Oil, series 27-S (HC 27-S). The request was generated after a pump diaphragm failed and a manned hyperbaric chamber became contaminated with HC 27-S. This HC 27-S is a polymer of chlorotrifluoroethylene (CTFE) and is used as a lubricating oil for pumps employed in hyperbaric chambers. In addition, HC 27-S is used to lubricate all O-rings on doors and service locks of the chambers.

Although no specific toxicity information is available on the polymers of CTFE, inhaled monomeric CTFE has been shown to produce renal damage in rats. The principal insult in the kidney was necrosis of the pars recta, and to a lesser extent, the pars convoluta of the proximal tubule. Signs of nephrotoxicity included diuresis, increased urinary lactate dehydrogenase (LDH) activity, serum creatinine, and blood urea nitrogen (BUN) with a concurrent decrease in urine osmolality. Water intake was increased by 25% in exposed rats (Potter et al., 1981). CTFE is metabolized to inorganic fluoride, which is excreted in the urine. In subchronic studies, tubules underwent regeneration, and necrosis was minimal upon further exposure, suggesting adaptation to CTFE toxicity (Buckley et al., 1982). The authors speculated that metabolism and/or disposition of CTFE was altered, or that regenerating tissue was refractive to CTFE toxicity.

The rat was selected as the test species to allow for comparison with the CTFE studies mentioned above. The numbers of animals per test group were kept to the minimum necessary for appropriate statistical analysis. These studies were performed as a preliminary assessment of the toxicity of acute and subchronic exposure to HC 27-S, taking into consideration the toxicity reported for the parent monomer CTFE.

### MATERIALS

#### Test Material

The HC 27-S test material (Table 11.2-1) was supplied by the NMRI/TD.

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* NMRI/TD Wright-Patterson Air Force Base, OH.

**TABLE 11.2-1. TEST MATERIAL DATA[a]**

| Item | HC 27-S |
|---|---|
| MILSPEC | MIL-L-24574 |
| Federal stock no. | 9150-01-101-8835 |
| Batch no. | 86-75 |
| NMRI/TD no. | 86-295-1 |
| Manufacturer | Halocarbon Products Corp., 82 Burlews Ct., Hackensack, NJ |
| Stability | Decomposes at temperatures above 260°C |
| Vapor pressure | Less than 0.01 mm Hg at 27°C |
| Additives | Organic acid rust inhibitor (0.1%) |
| Specific gravity[b] | 1.9225 at room temperature |

a Data Supplied by NMRI/TD
b Determined empirically

## Animals

Male and female Fischer 344 rats, weighing between 180 and 220 g and between 150 and 200 g, respectively, were purchased from Charles River Breeding Labs of Kingston, NY. Quality control assessments, conducted during a two-week quarantine period, showed the animals to be in acceptable health.

The rats involved in the acute assay were group housed (two to three per cage) in plastic cages with wood-chip bedding. Rats used in the subchronic assay were housed individually except during the quarantine period and the 14-day holding period postdosing. Prior to treatment, the rats were put into Nalgene metabolism cages for a one-week acclimatization period. All rats were fed Purina Formulab 5008 (food in metabolism cages was powdered) and deionized distilled water *ad libitum*, with the exception that rats were fasted for 16 h prior to oral dosing. Ambient temperatures were maintained at 21 to 25°C except for three deviations of short duration that were caused by failures in the building heating and air conditioning system. At those times, the temperatures ranged from 13 to 34°C. The light/dark cycle was set at 12-h intervals.

## METHODS

### Acute Assay (Oral LD$_{50}$)

The rats were fasted for 16 h prior to the administration of the oral dose. Five male and five female Fischer 344 rats were dosed with 5.0 g/kg. The HC 27-S was administered as neat agent after

the rats were individually weighed to determine dose volume. The rats were then maintained for 14 days of observation.

The rats were weighed at 1, 4, 7, 10, and 14 days postdosing and were observed at least twice a day for any signs of toxicity. At the end of the 14-day observation period, all rats were sacrificed for gross pathological examination.

*Subchronic Assay*

The subchronic study involved repeated dosing at one-half the $LD_{50}$ determined in the acute study. Since no deaths occurred at the acute limit test, the dose level was set at 2.5 g/kg. Six groups of Fischer 344 rats were dosed daily, including weekends, by gavage. Each test group received the same daily dose, but the groups were sacrificed at different times. Each control group received an equal volume of distilled water and was sacrificed with its corresponding test group (Table 11.2-2). The HC 27-S was administered as neat agent, and the volumes administered were calculated from the individual body weights and adjusted daily. Dosing was performed at the beginning of each day, prior to 0930 h. Food, provided following gavage, was removed at 1630 h each day. This study was initiated using female rats, followed by an identical regimen using male rats.

Body weights were measured daily throughout the study. Water consumption and urine output were measured gravimetrically each day during the dosing period. Urine samples taken one day predosing, on days 1, 3, 5, 7, 14, and 21 during dosing, and on day 14 postdosing, were analyzed for the parameters listed in Table 11.2-3.

TABLE 11.2-2. EXPERIMENTAL DESIGN OF SUBCHRONIC STUDY

|   | Dose Level | No. of Animals | Days on Test[a] | Days[a] at Sacrifice | Clinical Tests | Gross/ Histopathology |
|---|---|---|---|---|---|---|
| 1 | Control | 6 | 7 | 8 | yes | yes |
| 2 | $LD_{50}/2$[b] | 6 | 7 | 8 | yes | yes |
| 3 | Control | 6 | 21 | 22 | yes | yes |
| 4 | $LD_{50}/2$[b] | 6 | 21 | 22 | yes | yes |
| 5 | Control | 6 | 21 | 36 | yes | yes |
| 6 | $LD_{50}/2$[b] | 6 | 21 | 36 | yes | yes |

a Consecutive days including weekends.
b Since no deaths occurred at the limit test, the dose was 2.5 g/kg.

TABLE 11.2-3. URINALYSIS PARAMETERS ASSESSED IN THE SUBCHRONIC STUDY

| Assay Method | Parameters |
|---|---|
| N-Multistix (Ames) | Protein<br>Ketone<br>Bilirubin<br>Urobilinogen<br>Hemoglobin<br>pH<br>Glucose |
| Refractometer | Specific Gravity |
| DuPont Automated Clinical Analyzer (ACA) Picric Acid | Creatinine |
| Sigma Reagent Kit and Clinical Biological Analyzer Systems (COBAS) Bioassay | Serum glutamic-oxalacetic transaminase (SGOT)<br>N-Acetyl glucose aminidase (NAG)<br>Lactic dehydrogenase (LDH)<br>Calcium |
| Fluoride specific electrode[a] | Inorganic fluoride |

a Neefus et al. (1970).

The rats were fasted 16 h prior to sacrifice by $CO_2$ inhalation. Groups 1 and 2 were sacrificed on the morning following the 7th day of dosing; groups 3 and 4 were sacrificed on the morning following the 21st day of dosing; and groups 5 and 6 were sacrificed 14 days after the completion of dosing. At the time of sacrifice, blood was collected from the posterior vena cava for whole blood and serum analyses (Table 11.2-4). The following organs were weighed: heart, pituitary, liver, spleen, thymus, kidneys, testes, ovaries and brain. A gross pathologic examination was performed on each rat, and specified tissues were collected and prepared for histopathologic examination (Table 11.2-5). The right femur was freeze-dried in preparation for x-ray elemental analysis.

TABLE 11.2-4. ANALYSIS OF BLOOD AND SERUM SAMPLES FROM THE SUBCHRONIC STUDY

| Sample Type | Assay Method | Parameters |
|---|---|---|
| Whole blood | Coulter Counter | White blood cell (WBC) count<br>Red blood cell (RBC) count<br>Hemoglobin<br>Mean corpuscular volume (MCV)<br>Mean corpuscular hemoglobin (MCH)<br>Hematocrit<br>Mean corpuscular hemoglobin concentration (MCHC)<br>Differential leucocyte count |

(continued)

TABLE 11.2-4. (Continued)

| Sample Type | Assay Method | Parameters |
|---|---|---|
| Serum | COBAS | Glucose |
| | | Creatinine |
| | | Blood urea nitrogen (BUN) |
| | | Alkaline phosphatase |
| | | Creatinine phosphokinase (CPK) |
| | | SGOT |
| | | Albumin |
| | | Total Protein |
| | | Gamma gutamyl transpeptadase (GGTP) |
| | | Calcium |
| Plasma | Fluoride specific electrode[a] | Inorganic fluoride |

[a] Singer and Ophaug (1979).


TABLE 11.2-5. TISSUES HARVESTED FOR HISTOPATHOLOGY FROM THE SUBCHRONIC STUDY

| | | |
|---|---|---|
| Nose | Uterus[b] | Thymus |
| Bone marrow | Jejunum | Adrenals |
| Parathyroid | Stomach | Kidneys |
| Heart | Colon | Prostate[a] |
| Spleen | Muscle (quadriceps, femoris) | Ovaries[b] |
| Mandibular and mesenteric lymph nodes | Bone (sternum and both femurs)[c] | Ileum-duodenum |
| Urinary bladder | Thyroid | Pancreas |
| Salivary glands | Lungs | Brain |
| Testes[a] | Liver | Nerve(sciatic) |
| Seminal vesicles[a] | Pituitary | Esophagus |
| | | Trachea |

a Males only.
b Females only.
c Left femur for light microscopy, right femur for scanning electron microscopy and X-ray analysis.

## STATISTICAL ANALYSIS

Statistical analyses of collected data will be as follows. A repeated multivariate analysis of variance with Scheffe pair-wise comparisons (Dixon, 1985; Barcikowski, 1983) will be used for body weights, water consumption, urine volume, and clinical chemistry data. A two-factorial analysis of variance with multiple comparisons will be performed for both blood parameters and organ weights (Dixon, 1985; Barcikowski, 1983). Histopathologic data will be analyzed using either Fisher's Exact Test or, if not valid, the Yates' corrected Chi-square test (Zar, 1974). A probability of 0.05 or less will infer a significant change from controls.

## RESULTS

Study data are being analyzed.

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## 11.3 ACUTE INHALATION TOXICITY OF BEL-RAY SYNCOM 1400

### E. Kinkead and V. Harris

#### INTRODUCTION

Bel-Ray Syncom 1400 is a synthetic lubricating fluid presently used in Naval operations. It is a highly water-soluble material with the major component being polyalkylene glycol. A minor component, which has been reported to cause eye and skin irritation, is tert-octyl-n-phenyl-1-naphthylamine (MSDS, Ciba-Geigy Corporation). The manufacturer of alkylammon-ium-mixed alkyl acid phosphate, an additional component of Bel-Ray Syncom 1400, has warned that prolonged contact may result in skin irritation (MSDS, E.I. Du Pont deNemours & Co., Inc.).

Irritation and sensitization studies on Bel-Ray Syncom 1400 have been conducted (Gaworski, 1985) and minor eye and skin irritation reported at 4 and 24 h respectively. However, signs of irritation had resolved by the next evaluation period (24 and 48 h). Bel-Ray Syncom 1400 produced no sensitization response in guinea pigs. Acute inhalation toxicity information was not available.

The Naval Air Engineering Center began operational testing with the Bel-Ray Syncom 1400 fluid. Personnel working in a compartment where the fluid was being used complained of headaches and dizziness, and attributed the effects to the fluid. The fluid had been added to an open water tank at a concentration of 9% (by volume). Also present in the water was 0.2% of a 50/50 mixture of sodium nitrite and an emulsifier, sodium alkylsulfonylglycine. The air in the compartment was saturated with mist and steam, which may have contained the water-soluble Bel-Ray Syncom 1400.

To complement the available data on the acute toxicity of this fluid, the Naval Medical Research Institute/Toxicology Detachment (NMRI/TD) requested that acute inhalation toxicity tests be performed on an aerosol containing 9% Bel-Ray Syncom 1400 in water. Also present in the water would be a 0.2% weight/volume mixture of sodium nitrite and sodium alkylsulfonylglycine. As recommended by the Environmental Protection Agency, male and female rats were selected as the test animals for the inhalation tests. Existing alternative methods to animal testing are inadequate for this study.

### MATERIALS AND METHODS

#### Test Materials

Bel-Ray Syncom 1400 and the 50/50 mixture of sodium alkysulfonylglycine-sodium nitrite were supplied by NMRI/TD. The components of Bel-Ray Syncom 1400 are listed below.

| Material | Approx. % Mass |
|---|---|
| Polyalkylene glycol | 65.0 |
| Ester (unidentified) | 26.0 |
| Sulfated castor oil | 5.0 |
| Carboxy, acid salt | 2.5 |
| Tert-octyl-N-phenyl-1-naphthylamine | 0.5 |
| Organic phosphoric acid ester | 0.5 |
| Alkyl ammonium mixed alkyl acid phosphate | 0.3 |

The test material was prepared by adding 0.2% (wt/vol) sodium alkylsulfonylglycine-sodium nitrite mixture to a 9% solution (vol/vol) of Bel-Ray Syncom 1400 in deionized water.

## Animals

Male and female Fischer 344 rats weighing 225-250 g and 150-175 g, respectively, were purchased from Charles River Breeding Labs, Kingston, NY. The rats were subjected to a two-week quarantine period during which in-house health checks were conducted. Rats were group-housed in clear plastic cages with wood-chip bedding except for the 4-h exposure period, when they were housed in wire-bottom stainless-steel cages. Water and feed (Purina Formulab #5.008) were available ad libitum except for the exposure period. Animal room temperatures were maintained at 21° to 25°C, and the light/dark cycle was set at 12-h intervals.

## INHALATION EXPOSURES

### Aerosol Generation

The aerosol generation system consisted of a 250 mL, three-neck round-bottom flask containing a single six-jet Collison (BGI, Inc., Waltham, MA 02154) nebulizer. A peristaltic pump provided the test material to the nebulizer from a continuously renewed reservoir. The desired aerosol concentration was maintained by regulating pressure and airflow through the nebulizer.

### Concentration Analysis and Aerosol Characterization

Total chamber concentration was based on both gravimetric measurements and saturated water vapor concentrations. Metricel (Gelman Sciences, Inc., Ann Arbor, MI 48106) membrane-filter samples were taken during the study for gravimetric measurements of the nonvolatile constituents. Relative humidity and temperature were measured during the study since the primary component of the Bel-Ray Syncom atmosphere was water (91%). Calculations based on saturated water vapor were performed to derive the concentration of water vapor during the exposures. Similiar measurements taken in the control chamber provided baseline values of the water vapor contributed by the input air and the animals' expired breath. The total chamber concentration

(contaminants plus water vapor) less the background water vapor input constituted the reported concentration.

The aerosol in the exposure chamber was characterized using a Lovelace Multijet Cascade Impactor (Intox Products, Albuquerque, NM). This instrument provided a sharp collection efficiency and had well-defined effective cut-off ranges per stage. Six samples were taken during each 4-h exposure.

### Exposure Regimen

Five male and five female rats were exposed in a 250-L Wahmann (Hazleton Systems, Inc., Aberdeen, MD) inhalation chamber. Airflow through the chamber was equivalent to 15 chamber volumes/h. The chamber was maintained at a slight negative pressure to prevent contaminents from leaking into the room. Similiar groups of rats were exposed to air alone with identical parameters to provide background data for concentration analysis and comparison of body weights. Rats were weighed immediately prior to exposure and on days 7, 10, and 14 postexposure. All animals were sacrificed following the 14-day observation period for gross pathologic examination.

### Statistical Analysis

Body weight ± standard error of the mean (S.E.M.) was calculated according to Dixon (1985). Body weights were compared using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A probability of $<0.05$ inferred a significant change from controls.

### RESULTS

Five male and five female rats were exposed by inhalation to a mean aerosol concentration of 5.4 mg/L for 4 h. The mass median aerodynamic diameter of the particles was 1.4 $\mu$m, with a standard geometric deviation of 2.5 $\mu$m, well within the respirable range.

No signs of toxic stress were noted during the 4-h exposure period, nor did any deaths occur during the 14-day observation period. Mean body weight gains of the exposed rats during the postexposure period compared favorably with the mean body weight gains of the control group (Figure 11.3-1). Gross pathologic examination of all rats following the 14-day observation period revealed no exposure-related lesions.

### DISCUSSION

Four-hour inhalation exposure of male and female rats to a Bel-Ray Syncom 1400 hydraulic fluid-water solution resulted in no visible signs of toxic stress or deaths. Under the conditions of this test, this lubricating fluid-water solution does not appear to pose an inhalation hazard.
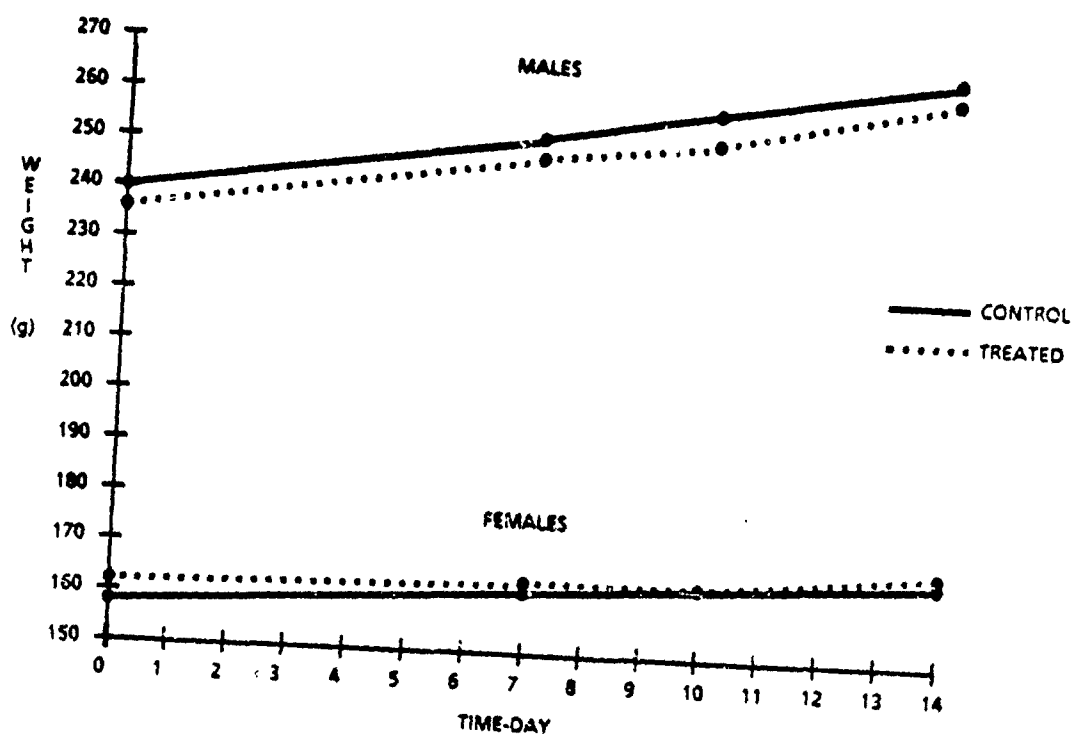
Figure 11.3-1. Body Weights for Test and Control Male and Female F-344 Rats.

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H. Leahy, H. Higman, and E. Kinkead

## INTRODUCTION

In addition to long-term research studies, emergency chemical analysis and toxicological evaluations of materials of interest to the Navy and Air Force are often incorporated into the research program. These studies are most often related to unanticipated exposures of personnel or environmental accidents which have the potential for exposure of civilian and military personnel. Results are usually used to determine whether more comprehensive testing of materials should be conducted or to define the potential risk associated with a particular incident.

Naval personnel requested that several analyses be performed to determine the potential neurotoxicity of a hydraulic fluid involved in an exposure incident aboard an aircraft carrier. Materials involved were related to phosphate triester compounds. Previous studies by this laboratory had determined the neurotoxicity of two of these materials, Fyrquel and Durad. Fyrquel studies provided negative results for neurotoxicity, whereas studies on Durad gave positive neurotoxic indications (Salomon et al., 1986).

Samples as received from the Navy consisted of two drum samples and materials collected from the forward and aft hydraulic lifts of the carrier. These materials were to be evaluated by a two-stage process: chemical analysis of the materials according to military specifications for total o-cresol. followed, if indicated, by a neurotoxic evaluation using adult chickens. This section reports the chemical anlayses performed to evaluate the need for further neurotoxicity tests based on the concentrations of o-cresol in the fluids and the patterns obtained for free phenols obtained by gas chromatography (GC) of the saponified ether extractables from the test materials as compared to known standards.

## MATERIALS

### Test Agents

All test materials were supplied by the Navy as received from the ship involved in the incident. The identification of the materials is provided in Table 11.4-1.

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## TABLE 11.4-1. IDENTIFICATION OF ANALYZED HYDRAULIC FLUIDS

| Sample # THRU | Naval Identification | Sample Label Identification |
|---|---|---|
| 907-01 | Hydraulic fluid original container FMC Corp. | MIL-H-19457 C (ships) NSN 9150-01-113-2047 07 May 87/GLH |
| 907-02A[a] | Hydraulic fluid original container Stouffer Chemical | MIL-H-19457 C (ships) NSN 9150-01-113-2047 07 May 87/GLH |
| 907-03 | Hydraulic fluid | Aft HPU sliding block and component lift #1 |
| 907-04 | Hydraulic fluid | Forward HPU sliding block and component lift #2 |

[a] This material contained two discrete layers as received. The upper layer was oily and was designated 907-02A. The lower layer, designated 907-02B, was an aqueous layer.

## METHODS

Sample preparation for the test materials and phosphate triester materials was in accordance with MILSPEC MIL-H-19457(SH). Phosphate triester materials are saponified overnight in a Parr Bomb at 140 to 150°C for 16 h. This saponification converts the ester groups to free phenolic compounds. After neutralization, free phenols are extracted from the sample with anhydrous ethyl ether and analyzed by GC to determine the amount of o-cresol present and to obtain characteristic GC patterns for comparison to known hydraulic fluids. The latest military specification for o-cresol content lists less than 1% total tri-o-cresol phosphate (TOCP) equivalent as acceptable for use in shipboard applications. Neurotoxic potential is calculated from the animal-study data.

Standards of o-cresol were prepared from 99 + % o-cresol obtained from Aldrich Chemical. Standard curves for o-cresol were prepared by dilutiing this material in ethyl ether over an appropriate working range then using area counts obtained from GC analysis. Ether extracts of the saponified sample materials were analyzed using identical chromatographic conditions (Table 11.4-2). The total o-cresol was quantified by comparison to the standard curves and reported as the percent of the total sample material by weight.

## TABLE 11.4-2. CHROMATOGRAPHIC CONDITIONS USED FOR ANALYZING O-CRESOL

| Instrument | Varian 1200 |
|---|---|
| Column | Carbopak "C" 0.1% SP-1000, 80-100 mesh |
| Carrier gas | Helium at 20 mL/min |
| Column temperature | 220°C |
| Injector temperature | 240°C |
| Detector temperature | 280°C |
| Attenuation | Range 1X, Attn 1X on the HP 3388 Integrator |
| Injection volume | 1 µL |

## RESULTS

Analysis of the shipboard materials indicated that the materials from the lifts, designated 907-03 and 907-04, contained o-cresol, as did the material provided in the drum sample designated 907-01. Materials from the layers in the second drum sample, designated 907-02A and 907-02B, did not contain o-cresol. The three samples containing o-cresol gave characteristic GC patterns consistent with those obtained from reference phosphate triester materials.

Analysis of the ether extract of saponifiable phenols from the 907-01 material indicated low concentrations of o-cresol, in the range of 0.006%. Extracts from the aft and forward hydraulic lifts, 907-03 and 907-04, respectively, had concentrations of o-cresol five- to tenfold greater than the material from the 907-01 container, and their GC patterns were similar to the phosphate triester materials. Archive samples of Fyrquel and Durad had concentrations of o-cresol consistent with previous studies and characteristic phosphate triester patterns upon GC analysis.

Table 11.4-3 provides the concentrations of o-cresol expressed as percent tri-o-cresol phosphate obtained from the following MILSPEC calculation:

$$\text{weight \% } o\text{-cresol} = \frac{(\text{weight standard}) \times (\text{area sample [GC]}) \times 100}{(\text{area standard [GC]}) \times (\text{weight sample})}$$

### TABLE 11.4-3. PERCENT TRI-O-CRESOL PHOSPATE CONTENT OF ANALYZED HYDRAULIC FLUIDS

| Sample | % TOCP |
|--------|--------|
| 907-01 | 0.006 % |
| 907-02A | —a |
| 907-02B | —a |
| 907-03 | 0.031 |
| 907-04 | 0.063 |
| Fyrquel | 0.023 |
| Durad | 0.214 |

a No o-cresol detected in sample

Neurotoxic studies will provide the data set that ultimately determines conformance to the MILSPEC. However, chemical analysis of the materials determining the concentration of free o-cresol can be used to eliminate materials from the animal-testing study. Chemical data provides a rational basis for screening.

GC analysis provides a characteristic pattern for free phenols obtained from the ether extract of saponified test materials. These patterns were compared to results obtained from archive samples

of Fyrquel and Durad, hyrdraulic fluids known to contain o-cresol and a series of free phenolics. GC patterns obtained from analysis of shipboard samples and reference materials are shown in Figures 11.4-1 to 11.4-5.

## DISCUSSION

Results of the chemical analysis indicate that the materials obtained from the aft and forward hydraulic lifts, 907-03 and 907-04 respectively, contain o-cresol and give GC patterns similar to known o-cresol containing materials. The container samples identified as FMC and Stouffer Chemical samples were analyzed as received. The sample labeled FMC (907-01) was similar to Fyrquel which, incidentally, is manufactured by Stouffer. The sample labeled as Stouffer contained two discrete layers (907-02A and 907-02B) as received and did not yield chromatographic data consistent with that expected for a phosphate triester sample from either layer.

GC patterns obtained from the two hydraulic lift samples do not match either Fyrquel or Durad. Comparison to an overlay of both known materials indicates that the hydraulic lift samples may be a mixture of the two materials. Durad has been previously identified as having significant neurotoxic potential.

Additional neurotoxic studies involving adult chickens will be conducted on the shipboard samples. Appropriate specimens were ordered for the peroral administration and observation studies, which will supply the data required to complete the evaluation of the shipboard materials.

## REFERENCE

Salomon, R.A., T.R. Boosinger, R.H. Bruner, A.P. D'Addario, C.L. Gaworski, E.R. Kinkead, J.R. Horton, W.J. Bashe, R. L. Einhaus, D.L. Pollard, and J.D. Diaz. 1986. Comparative studies on the short-term toxicity of the hydraulic fluids MIL-H-19457C, MIL-H-19457B, and MIL-H-22072B. Report No. AAMRL-TR-86-030, Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH; NMRI-86-35; Naval Medical Research Institute, Bethesda, MD.
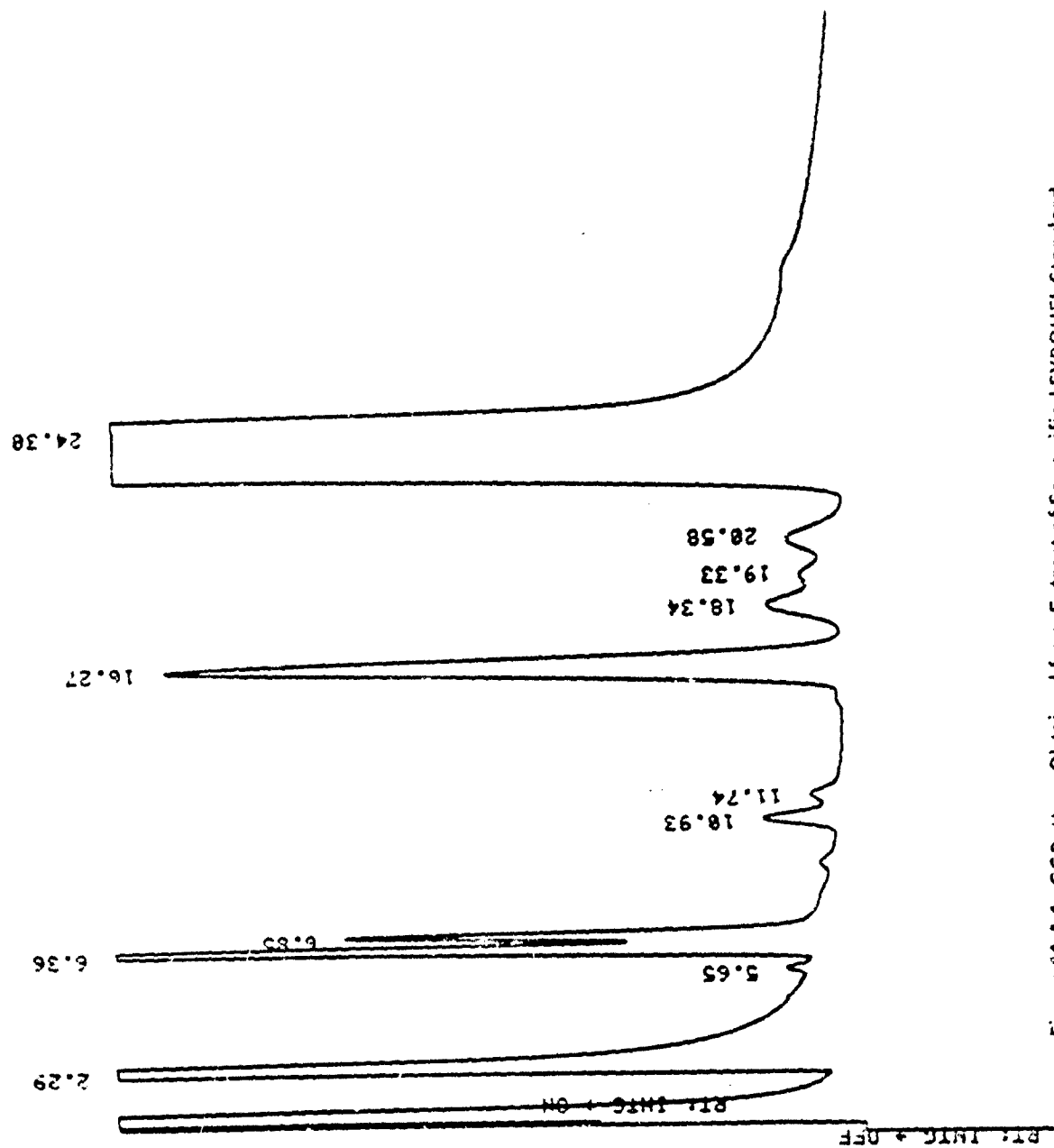
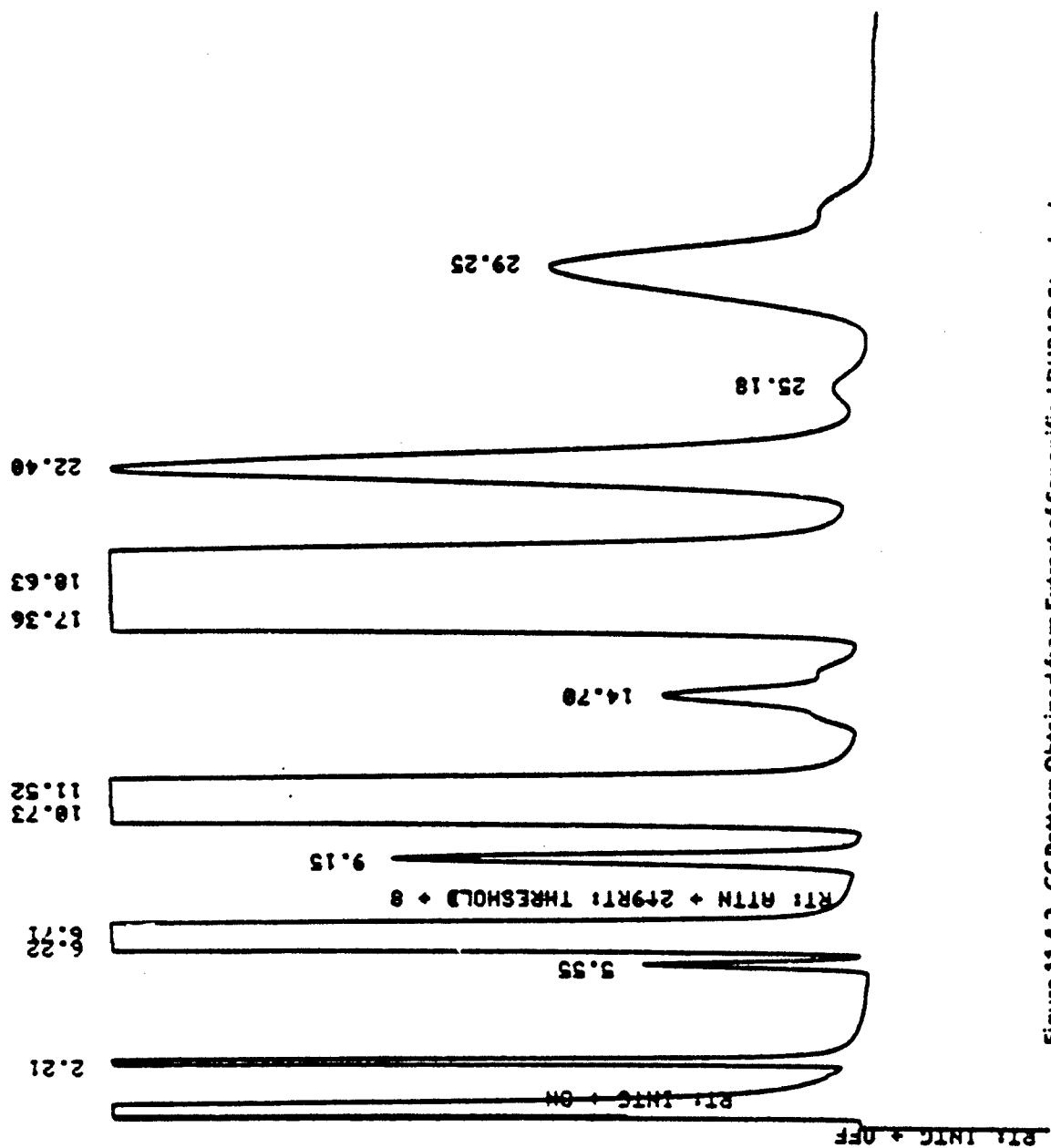Figure 11.4-1. GC Pattern Obtained from Extract of Saponified FYRQUEL Standard.

Figure 11.4-2. GC Pattern Obtained from Extract of Saponified DURAD Standard.
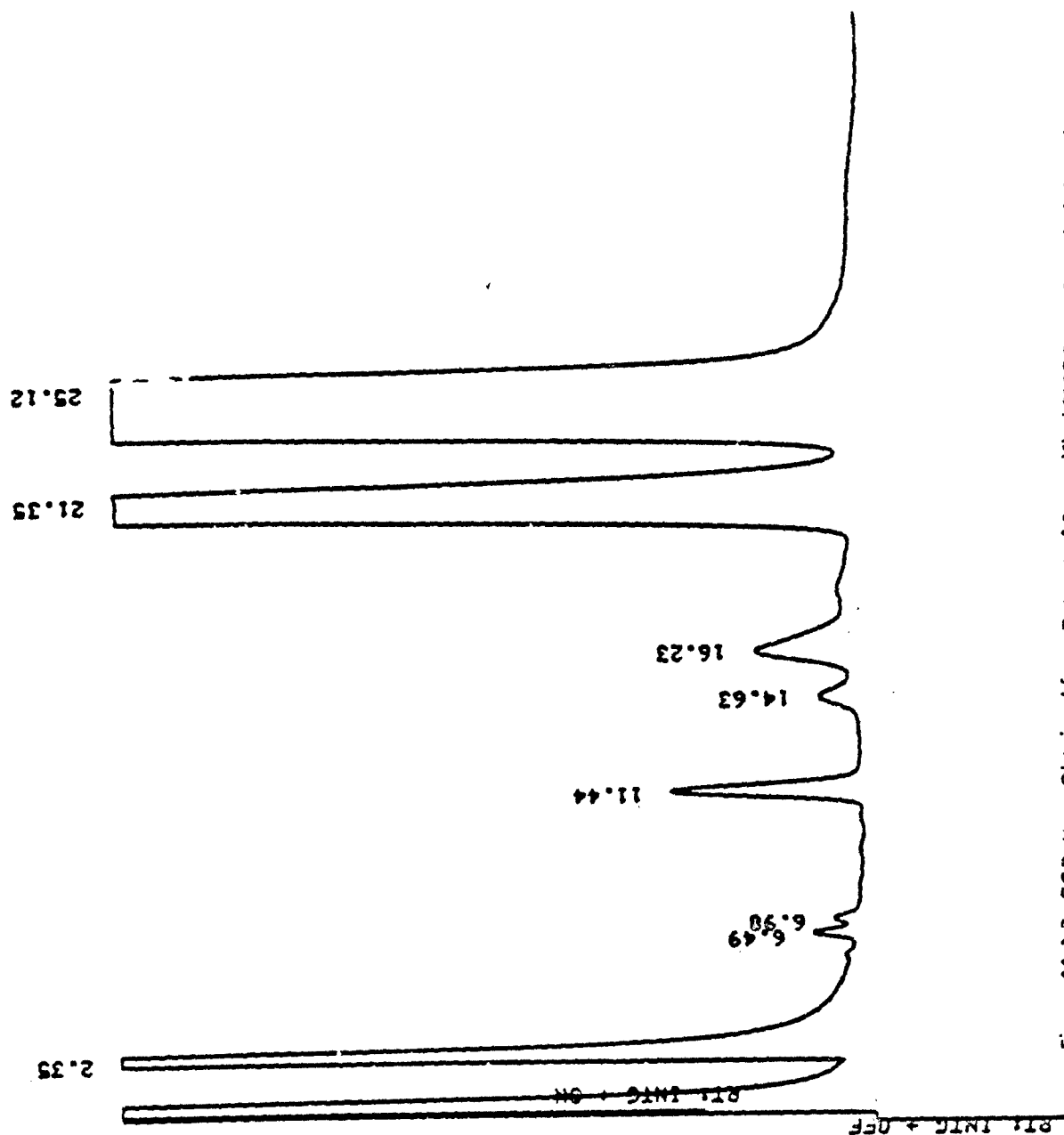
Figure 11.4-3. GC Pattern Obtained from Extract of Saponified FMC Drum Sample (907-01).
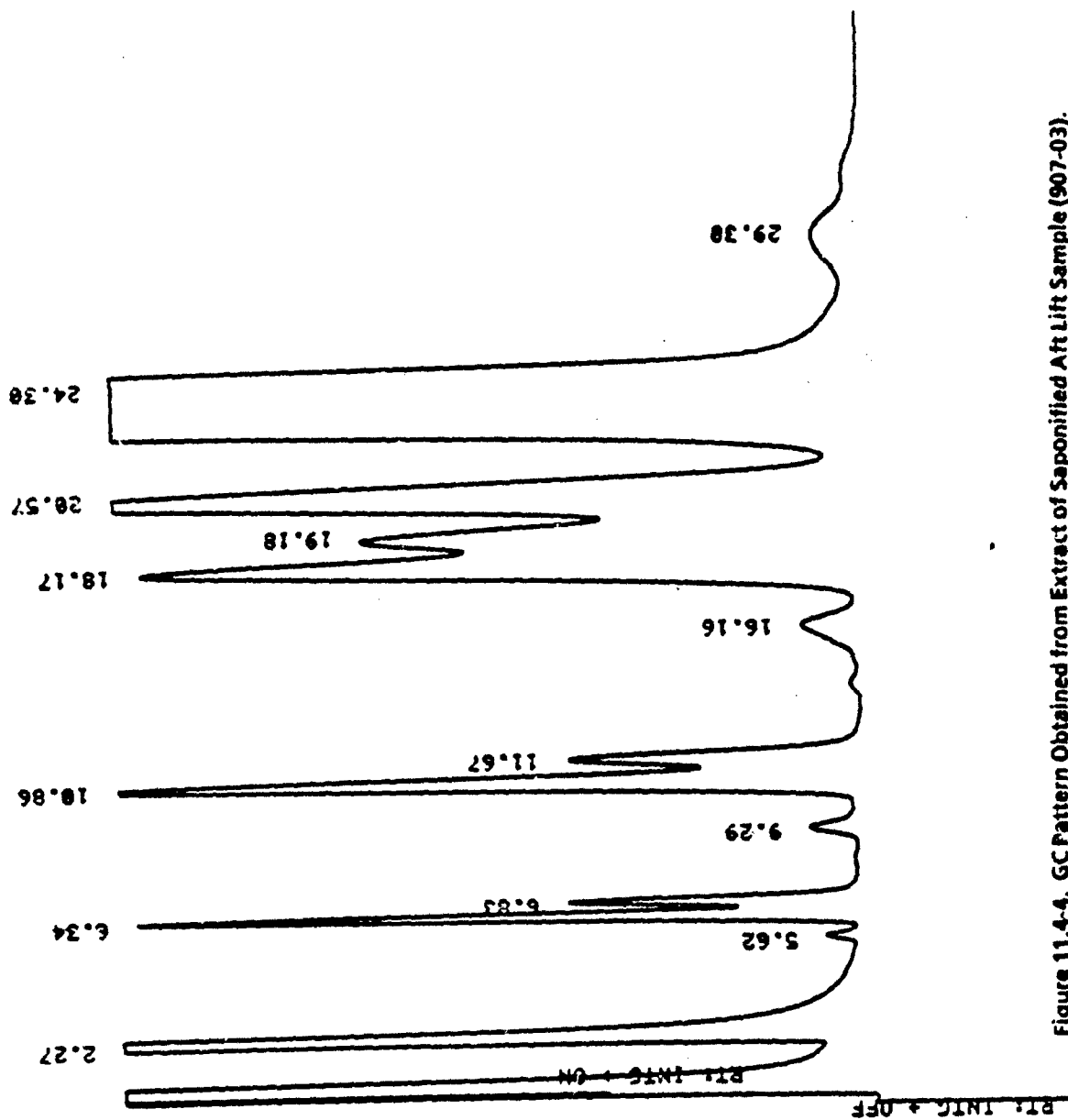
Figure 11.4-4. GC Pattern Obtained from Extract of Saponified Att Lift Sample (907-03).
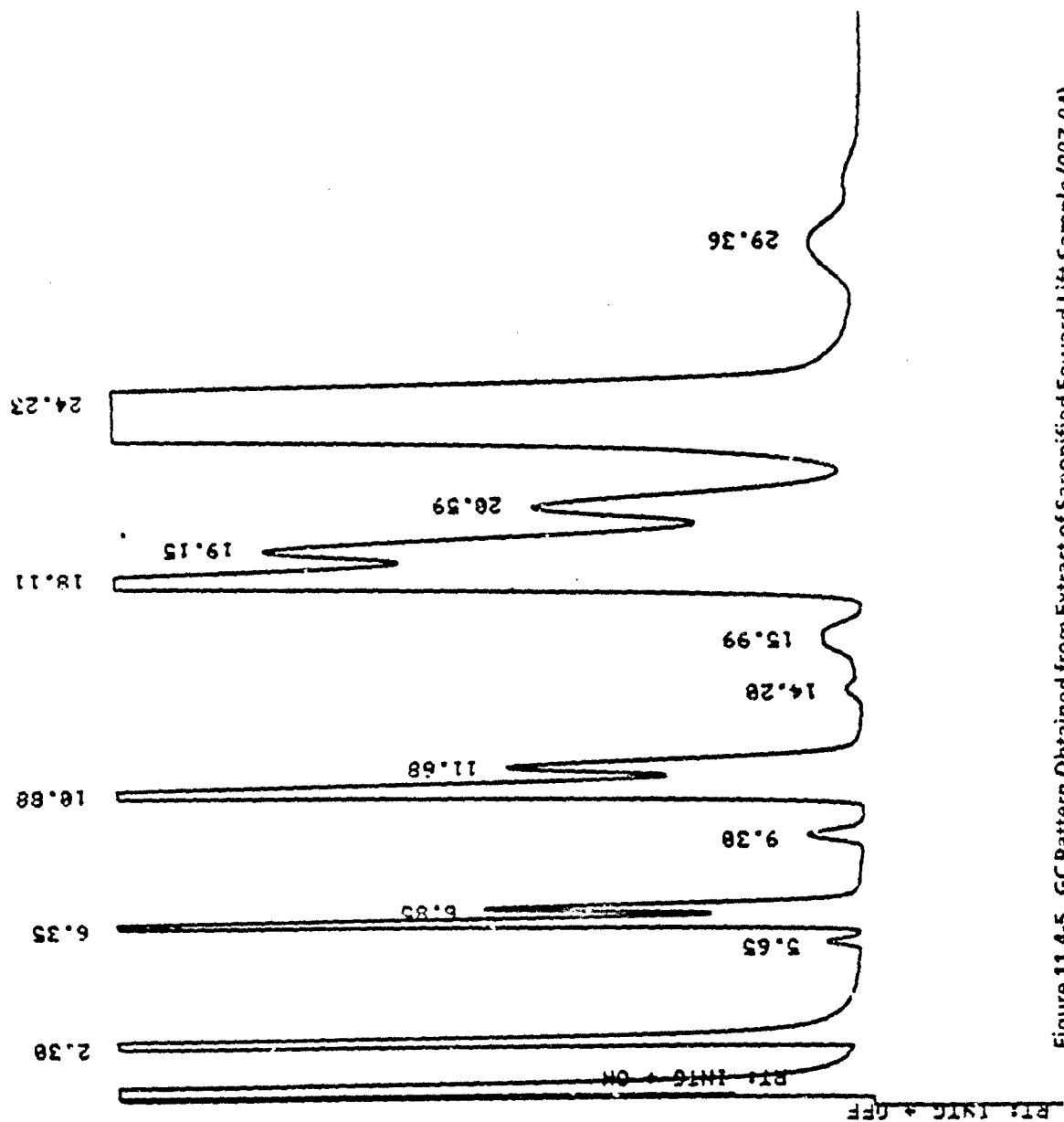
Figure 11.4-5. GC Pattern Obtained from Extract of Saponified Foward Lift Sample (907-04).

# SECTION 12

## EXPOSURE AND FACILITIES ENGINEERING

### 12.1 THE TOXIC HAZARDS RESEARCH UNIT GENER.   'NHALATION EXPOSURE FACILITY

E. Kimmel, R. Carpenter, and K. Yerkes

*INTRODUCTION*

A primary task of the Toxic Hazards Research Unit (THRU) engineering staff is to expand the capacity of and modernize the inhalation toxicology exposure test equipment located at the Toxic Hazards Division (TH) of the Armstrong Aerospace Medical Research Laboratory (AAMRL). The objective was to employ new exposure system technology to provide TH with exposure capabilities that are flexible in application while remaining cost effective. The overall system required was one that could be configured readily to conduct several different short-term, acute inhalation toxicology studies using approoriate numbers of small animals with effective expenditure of manpower, yet also be suitable for the conduct of long-term, chronic inhalation studies with relatively large numbers of small animals. Energy efficiency, durability, and economic utilization of limited laboratory space were also requirements of the exposure system. An additional requirement was the design of a system suitable for use with test materials of widely varying toxicity and physicochemical properties. The initial phase of this task was completed last year with the design of the THRU inhalation exposure chamber described previously (Kimmel and Carpenter, 1987). Six of these chambers were fabricated and have been placed into operation at the THRU. At present, the second phase has been completed; chamber ventilation and manual control systems were installed as well as a subsystem for automated collection of data. Plans for a third phase, feedback control of the exposure system, are under development.

*DISCUSSION*

*Exposure Chamber Ventilation System Design*

In principle, standard industrial ventilation practices can be applied to the development of exposure ventilation systems. However, there are unique and stringent design requirements for inhalation exposure chamber ventilation systems that are not normally included in the design of conventional ventilation systems. These include the prerequisite for an absolutely leak-free air distribution network; extensive air cleaning and decontamination capabilities; and extraordinarily fine control of system flow, pressure, temperature, and relative humidity (RH). Exposure ventilation systems differ from conventional ventilation systems in that the former operate at what are considered very low flow rates across a very wide system pressure differential. Ideally, each exposure

chamber should be ventilated independently of the others. However, the cost of individualized chamber ventilation is prohibitive, and the complexity of individualized chamber ventilation in a multichamber exposure system inordinately complicates system operation and maintenance. Hence, all of the THRU exposure chambers were ventilated via common air supply and exhaust manifolds.

The chamber ventilation system (Figure 12.1-1) was divided into the supply subsystem and the exhaust subsystem, both of which were provided with active movement of air by centrifugal fans. The supply subsystem is a recirculating air system ducted to the inlet of each chamber with an open port for make-up air supply. Flow through the open port is controlled by a throttle valve to maintain a constant pressure head (5 to 7 in. of $H_2O$ positive with respect to ambient) in the supply duct under varying conditions of flow demand. Therefore, adjustments of flow and pressure can be made in any given exposure chamber with minimal influence on other chambers in the system. Total system flow and pressure is adjusted through the open port. Included in the supply air subsystem are a prefilter and an absolute (HEPA) filter for removal of unwanted particles from the supply air and provisions for removing unwanted vapors from the supply air can be included when necessary. Under normal operating conditions, flow through the exposure chambers is provided by the supply subsystem. Airflow into each chamber is controlled by an inlet control valve and is measured with an orifice meter.

Pressure in each of the exposure chambers, relative to the room, is determined by the exhaust subsystem, which is maintained at 15 to 20 in. $H_2O$ subambient pressure. The exhaust subsystem is not a recirculating system, but like the supply system, has an unoccupied port for make-up air supply. Hence, by controlling the proportion of air supplied through the open port via a throttling valve, total flow and pressure can be kept constant in the exhaust subsystem regardless of chamber demand. Flow out of each chamber is controlled via the chamber exhaust valve and measured by an orifice meter on each chamber. The exhaust from each chamber passes through a HEPA filter to remove particles. Charcoal filtration for removing vapors can be added as necessary. After filtration, the exhaust air from all of the chambers is passed through water misting scrubbers prior to release into the external environment.

The total pressure differential maintained across the chamber ventilation system is approximately 20 in. $H_2O$, and maximum total flow through the system is 100 standard cubic feet per minute (SCFM). Thus, under full demand conditions (all six chambers operating simultaneously) the system is capable of stable operation at individual chamber flows up to 15 SCFM, which is twice the normal operational maximum, and subambient chamber pressures well beyond the normal operating pressures of 1 to 2 in. $H_2O$. This reserve capacity, in conjunction with the ability to balance the supply and exhaust subsystem pressure differentials and flows, allows for highly stable operation of the system. Temporary or long-term shutdowns of individual chambers or groups of chambers are possible without upsetting flow and pressure balance in chambers remaining in operation.
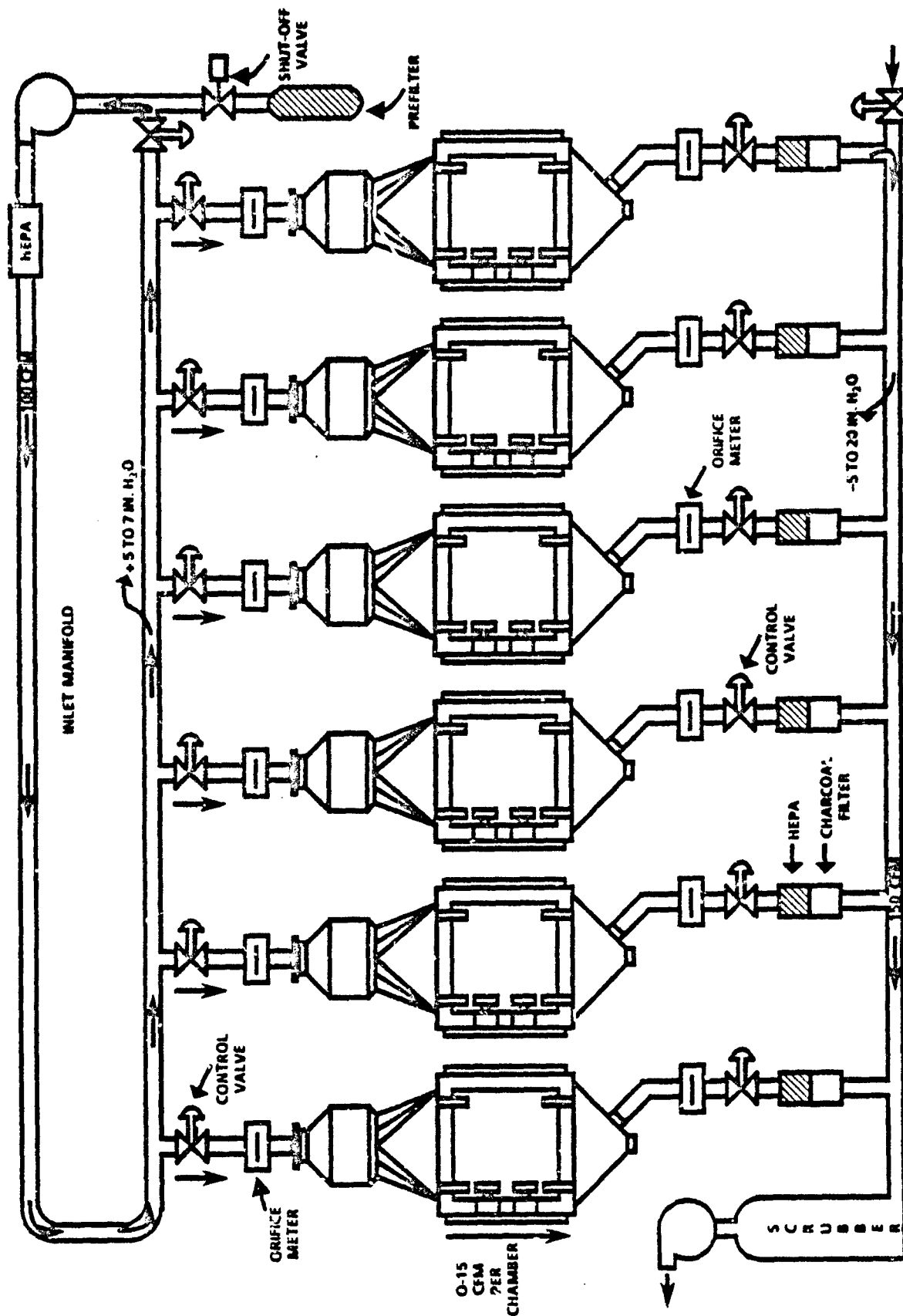
Figure 12.1-1. Schematic Diagram of the THRU Exposure Chamber Ventilation System.

SHUT-OFF VALVE

PREFILTER

HEPA

INLET MANIFOLD

+.5 TO 7 IN. H₂O

ORIFICE METER

-5 TO 25 IN. H₂O

CONTROL VALVE

HEPA

CHARCOAL FILTER

150 CFM

CONTROL VALVE

ORIFICE METER

0-15 CFM PER CHAMBER

SCRUBBER

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### Operational Support Systems

In addition to the primary ventilation system, the total exposure system is composed of several support systems. Distribution networks for breathing quality compressed air and vacuum were installed, providing several access points for both compressed air and vacuum supply to each chamber. These systems are essential for toxicant generation and sampling the chamber atmosphere; and a useful resource to support activities ancillary to chamber operation. An electrical distribution subsystem was installed, which provides three dedicated circuits for chamber operations. Each chamber was provided with eight separate electrical outlets to provide power for instrumentation.

An animal watering system was installed to provide each chamber with deionized water, allowing continuous housing of the animals during subchronic and chronic exposures. In addition to the animal watering system, each chamber was supplied with a source of steam-heated water for automatic washing and decontamination of the chambers between exposures.

Normal chamber operating parameters (flow in, flow out, chamber pressure, temperature, and RH) for each chamber are monitored continuously, and these data are recorded automatically via a microprocessor-based data acquisition system. When analytical methods and instrumentation permits, exposure data also are monitored continuously and recorded automatically.

### Design Goals

As noted above, the exposure system is in its second development phase and, although it is presently functional, several improvements for the system are planned. The current system does not have necessary capability for thoroughly conditioning the chamber supply air, particularly with regard to control of chamber temperature and RH. Chamber toxicant level is controlled manually by adjusting chamber airflow and generator output. Such manual control requires constant monitoring by exposure personnel. Furthermore, although the possibility of contaminating the work environment has been minimized by a series of pneumatically actuated shut-off valves used to isolate the exposure system, the system does not provide for emergency cleansing of the animal exposure atmosphere. Hence, potential exists for experimental compromise due to overdosing the animals during emergency situations, such as a physical-plant power failure or a temporary excursion of toxicant generator output. Plans for the next generation of the exposure system include measures to eliminate these system deficiencies.

The current system has a relatively simple data acquisition system, which, although functional, does not satisfy more sophisticated system-control requirements. The next generation of the exposure system will include control instrumentation linked to an expanded data acquisition system to provide feedback control of system operation. Accompanying this feedback control loop will be

the necessary generator and chamber flow bypass systems, which ensure safe operation of the system with minimal chance for premature termination of an experiment due to system malfunction. Generalized schematics, describing the proposed control and bypass systems for a single chamber, are shown in Figures 12.1-2 and 12.1-3. Included in the revised chamber ventilation system are provisions for chamber airflow bypass, generation system flow bypass, and dilution systems for both. Chamber operational parameters and exposure conditions will be monitored and recorded continuously and through a series of feedback loops. Coupled with automatic controls, these variables will be maintained automatically within specified limits. In addition, predetermined alarm levels will notify exposure personnel when out-of-limit conditions exist for any one of the monitored variables, thus permitting prompt correction of conditions that either have exceeded control setpoints or affect controlled parameters indirectly. Furthermore, the control system will have a self-diagnostic feature that will not permit the exposure system to start up when parts of the control system are not functional or are not set properly for the intended mode of operation.

In addition to providing for automatic monitoring and control of the exposures, the system will be configured to recognize several operational states (Figure 12.1-4). System operation in a given state or transition from one state to another will require recognition of numerous mutually inclusive and exclusive events. Hence safe operation of the system in each of the several operational modes will be ensured automatically unless manual override conditions are in force. Likewise, provisions will be included that allow operation of the exposure system under manual control when protocol requirements call for special or unique application of the exposure system.

The advantages of installing feedback control loops in the exposure system are obvious in terms of improved quality of control over the exposures, accuracy of data recording and, consequently, productivity of exposure personnel.

## REFERENCES

Kimmel, E.C. and R.L. Carpenter. 1987. The THRU whole-body inhalation exposure chamber. In: W.E. Houston, R.S. Kutzman, R.L. Carpenter, eds. *1986 Toxic Hazards Research Unit Annual Report.* Report No. AAMRL-TR-87-020, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-87-2, Bethesda, MD: Naval Medical Research Institute.

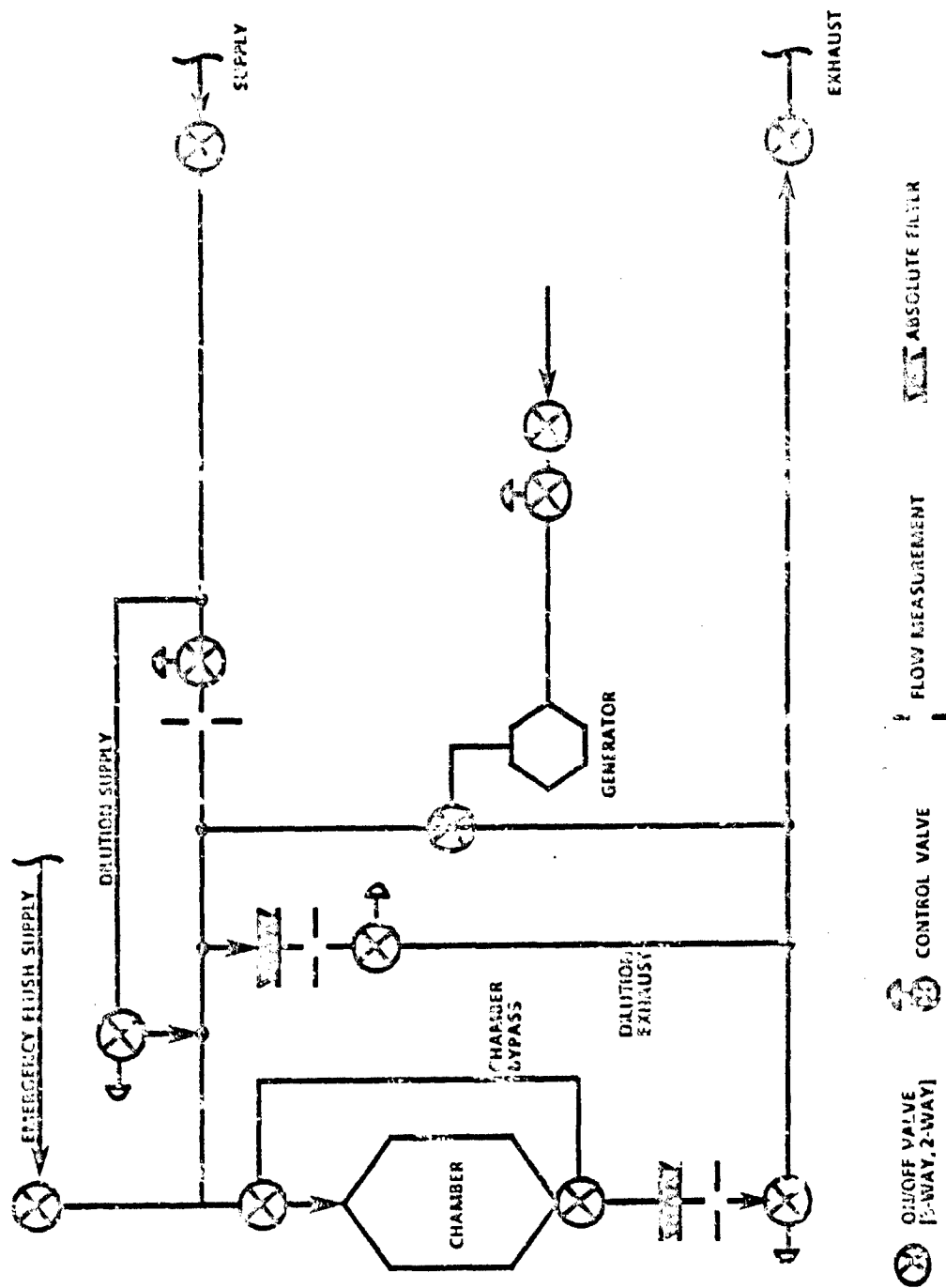Figure 12.1-2. Schematic Design of a Typical Exposure Chamber Ventilation and Bypass System.

SUPPLY

EXHAUST

GENERATOR

DILUTION SUPPLY

EMERGENCY FLUSH SUPPLY

CHAMBER BYPASS

DILUTION EXHAUST

CHAMBER

ON/OFF VALVE (3-WAY, 2-WAY)

CONTROL VALVE

FLOW MEASUREMENT

ABSOLUTE FILTER

SET POINTS
- CHAMBER FLOW
- CHAMBER PRESSURE
- CHAMBER CONCENTRATION

TEMPERATURE

% RH

CONCENTRATION

PRESSURES

FLOW

START/STOP

POWER OUT

HIGH CONCENTRATION

CHAMBER
MONITOR
AND
CONTROL

PRINTER AND MONITOR DISPLAY

PRESSURE, FLOW, TEMP, AND RH
DATA AND FEED BACK

CHAMBER FLOW

CHAMBER PRESSURE

DILUTION FLOW

GENERATOR OUTPUT

ALARM AND CORRECTIVE ACTION

NONEXPOSE

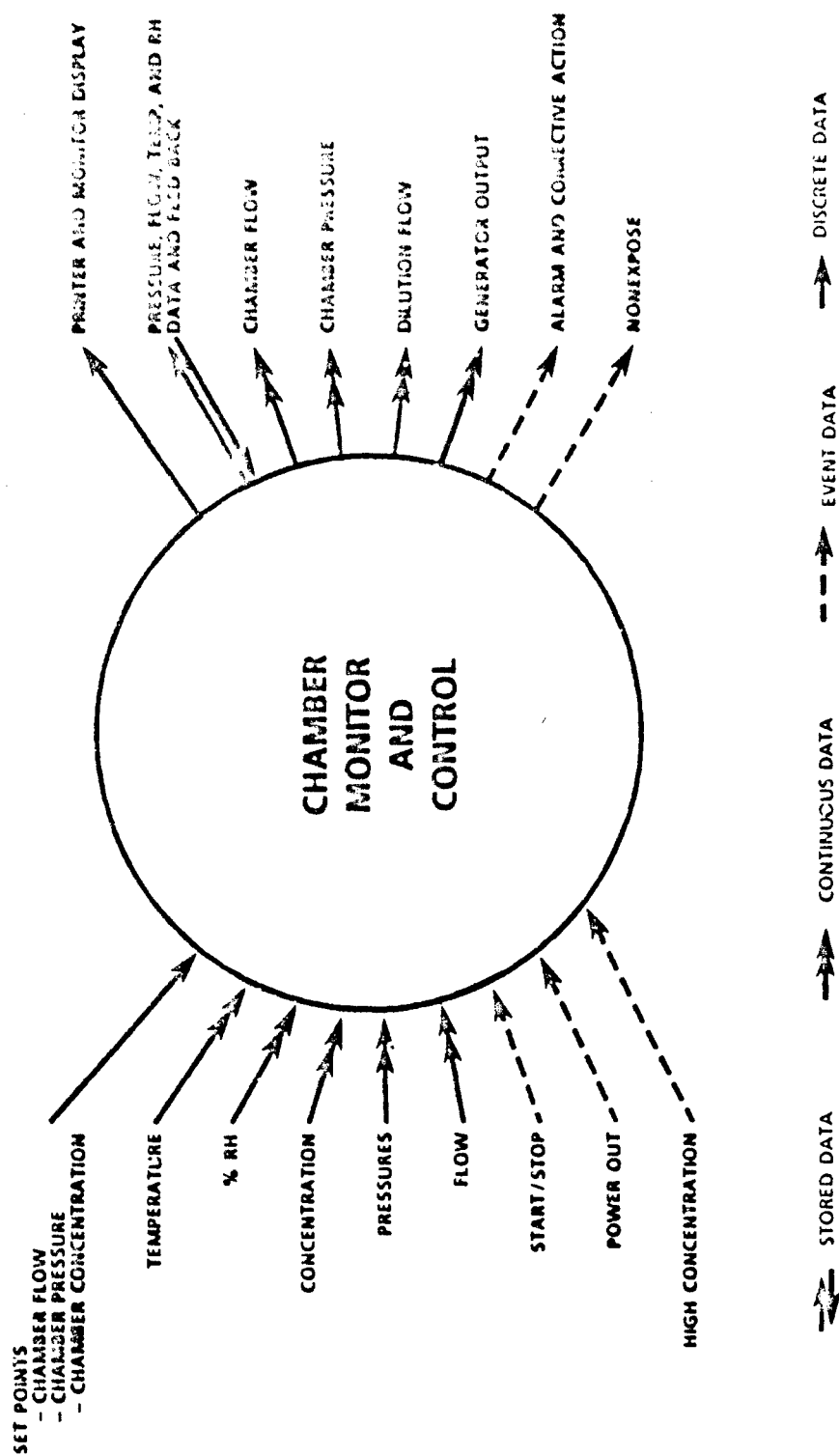STORED DATA      CONTINUOUS DATA      EVENT DATA      DISCRETE DATA

Figure 12.1-3. Schematic Design of a Typical Monitoring and Feedback Control System for a Single Chamber in Exposure Mode.

190
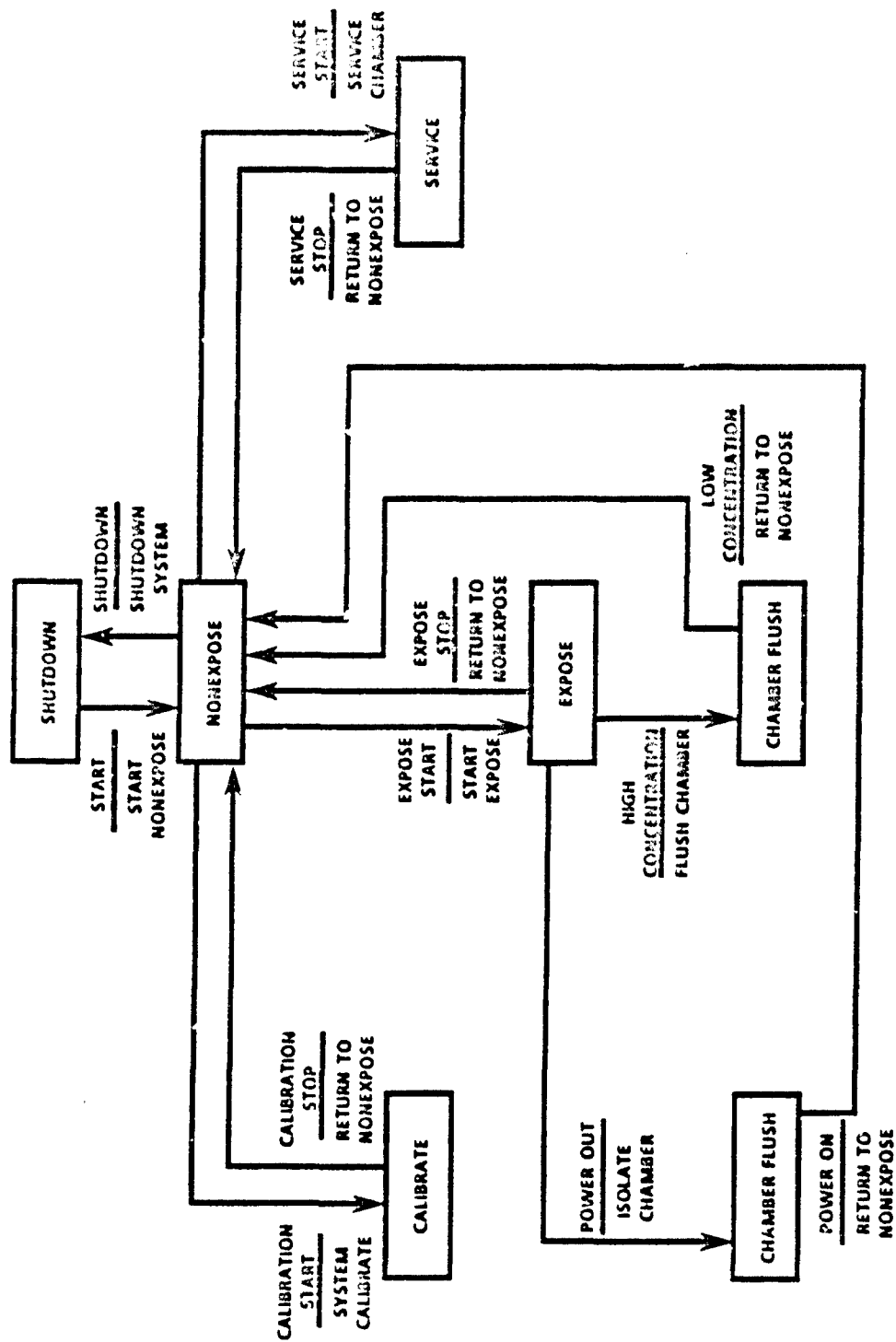
Figure 12.1-4. Exposure Chamber Operational State Transition Diagram.

## TOXIC HAZARDS RESEARCH UNIT ORGANIZATIONAL CHART

## TOXIC HAZARDS RESEARCH UNIT PERSONNEL LIST

### OFFICE OF DIRECTOR

W.E. (Ed) Houston, Ph.D.
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    Doam, Charles R.
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    Kimmel, Edgar C., Ph.D.
    Leahy, Harold F.
    Pollard, Daniel L.

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    Smutak, Donald A.
    Soloman, Kenneth G.
    Sonntag, William B.
    Stokes, James S.
    Yerkes, Kirk L.
    Young, James W.

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### SAFETY

Larry R. Snelling
Health & Safety Representative

### TOXICOLOGY

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    Bunger, Susan K.
    Culpepper, Brenda T.
    Deiser, Patricia A.
    Del Raso, Nicholas J.
    Dille, Susan E.
    Douthwaite, Martha S.
    Drerup, Joanne M.
    Godfrey, Richard J.
    Helton, C. Douglas
    Henry, Sandra S.
    Keaton, Karen M.
    King, Greg A.
    Kinkead, Edwin R.
    Malcomb, Willie J.
    McDonald, Gayle A.
    Neely, Gloria A.
    Nicholson, Jerry W.
    Reed, Yvonne M.
    Schimmel, Brenda D.
    Smith, Jessie L.
    Wagner, Sharon L.
    Williams, Deborah L.
    Wilson, Janet L.

# TOXIC HAZARDS RESEARCH UNIT
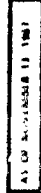
# APPENDIX B

## QUALITY ASSURANCE

Research programs at the Toxic Hazards Research Unit (THRU) are designed to be in compliance with the US Environmental Protection Agency's (EPA) 40 CFR, Part 792 "Toxic Substances Control; Good Laboratory Practice (GLP) Standards." A THRU-wide review was conducted to determine the Unit's compliance with these standards. Those areas of noncompliance, which were identified, are being addressed through a process of periodic management review and the implementation of the Quality Assurance Program within the THRU Research Programs.

Quality Assurance conducts procedure audits quarterly. Those audits conducted since the last Annual Report are listed below.

| TECHNICAL DIRECTIVE NUMBER | TEST PHASE | 1987 AUDIT DATE(S) |
|---|---|---|
| THB 0-004 | Nose-only exposure Expired air analysis | 20, 21 May 1987 |
| USN 0-007 | Inhalation limit tests | 26 Jun 1987 |
| USN 0-012 | Skin irritation | 13-16 Oct 1987 |
| USN 0-013 | 21-day inhalation | 9 Jul 1987 |
| | Repeat dermal | 25 Aug 1987 |
| USN 0-014 | Oral dosing | 16 Mar 1987 |
| | Specimen collection | 17 Mar 1987 |
| | Specimen analysis | 17 Mar 1987 |
| THA 0-017 | Interim data audit | 7 Oct 1987 |
| USN 0-018 | Inhalation limit test | 17 Jun 1987 |
| THA 0-020 | Repeated dermal | 9, 15 Jun 1987 |
| | Repeated oral | 20, 21, 26-28 May; 1-3, 12 Jun 1987 |
| | Skin sensitization | 6, 11, 13, 18 May; 3 Jun 1987 |
| | Oral dosing limit test | 30 Apr; 7, 14 May 1987 |

| TECHNICAL DIRECTIVE NUMBER | TEST PHASE | AUDIT DATE(S) |
|---|---|---|
| THA 0-020 | Skin irritation | 5, 7, 8 May 1987 |
| | Eye irritation | 12-15 May 1987 |
| THA 0-022 | Interim data and records audit | 21-25 Sep 1987 |
| THA 0-031 | Interim data audit | 7 Oct 1987 |
| THB 0-043 | Interim data audit | 7 Oct 1987 |
| THB 0-045 | 90-day inhalation | 14 Oct 1987 |
| | Skin sensitization | 21-23, 28-30 Sep; 5, 7, 9,12 Oct 1987 |
| | Skin irritation | 5-8 Oct 1987 |
| | Eye irritation | 20-23 Oct 1987 |

Comprehensive data and records audits were conducted at the completion of each study. The study report and data audits conducted since the last Annual Report are listed below.

| TECHNICAL DIRECTIVE NUMBER | REPORT TITLE |
|---|---|
| USN 0-007 | The evaluation of the acute toxicity of four water-in-oil hydraulic fluids. |
| TH 0-009 | Evaluation of the acute toxicity of four compounds associated with the manufacture of o-ethyl-o'-(diisopropylaminoethyl)-methylphosphonite. |
| THB 0-011 | Evaluation of the acute toxicity of selected-ground water contaminants. |
| USN 0-012 | The evaluation of the acute skin irritation of an "activated peroxide" preparation used for decontamination. |
| USN 0-018 | Evaluation of the acute inhalation toxicity of Bel-Ray Syncom 1400 – water solution. |
| USN 0-026 | Evaluation of the acute toxicity of a synthetic polyalphaolefin-based hydraulic fluid. |
| Work conducted by Former THRU Contractor | Evaluation of the inhalation and skin absorption kinetics of a cyclotriphosphazene-based hydraulic fluid. |

Work conducted by                Chlorotrifluoroethylene oligomer evaluation of acute
Former THRU Contractor           delayed neurotoxicity in hens, and study of absorption
                                 and metabolism in rats following oral, dermal and
                                 inhalation exposure.

# APPENDIX C

## SAFETY AND HEALTH PROGRAMS

The THRU expanded its safety programs to offer more services to its employees. This includes (1) tailoring a Medical Surveillance Program to the diverse groups of personnel, (2) starting an Emergency Medical Notification and Health Care Program using Air Force facilities, (3) inspecting facilities with Air Force personnel, (4) encouraging more personnel to participate in safety committee meetings, and (5) revising chemical inventories of THRU laboratories.

The THRU Safety Committee has taken a more active role in maintaining a safe work environment. A defined purpose and use of "problem solving" organizational methods have made this committee more effective. Each member of the committee represents an operational section of the organization.

Monthly Grounds Safety Inspections of the THRU areas were coordinated with Air Force safety monitors. This cooperation has allowed inspections to be expanded to include emergency lighting, self-contained breathing apparatuses, waste chemical storage, eyewashes, and safety showers.

The importance of the safety and health of THRU personnel conducting toxicology studies is being addressed. Safety practices and procedures are being developed for "Inhalation Exposure Studies" conducted in the General Toxicology Laboratory. These Guidelines establish "Safety Operating Procedures," necessary for safe operations. Some examples of the guidelines developed include "Emergency Evacuation Procedures," "Decontamination Procedures," and "Respiratory Protection Program."

## APPENDIX D

## ANIMAL HUSBANDRY TECHNICIAN TRAINING PROGRAM

NSI-ES Animal Husbandry technicians are responsible for the care of all animals housed in Buildings 79, 429, 433, 838 and 839 in Area B, W-PAFB. Responsibilities include animal care during quarantine and holding, as well as during the pre-and postexposure periods of the in-life phase of scientific studies. The diversified training of NSI-ES/THRU Animal Husbandry technicians allows for additional animal handling and laboratory support capability at AAMRL to support approved research protocols.

The number of THRU animal technicians currently certified by the American Association for Laboratory Animal Science (AALAS) are listed below:

Laboratory Animal Technologists  -  5

Laboratory Animal Technicians  -  4

Assistant Animal Technicians  -  1

During this year, two technicians were certified as Laboratory Animal Technicians, and one technician was certified as a Laboratory Animal Technologist. The outline of the AALAS course was described in detail in a previous report (MacEwen and Vernot, 1975). All references listed by AALAS that are utilized in preparing for examinations are available through the THRU and Air Force libraries. Two technicians are currently studying for the Assistant Animal Technician and Laboratory Animal Technician certification tests in November 1987.

Ongoing training and education for all of the technicians during the past year included

- Purina Laboratory Animal Care Course, completed by six new technicians.
- training for the Xybion system; attended by three technicians;
- Thomas Dome Chamber Maintenance Training, completed by nine technicians in support of inhalation studies;
- American Red Cross Basic Life Support Course (CPR) completed by four technicians; and
- in-house training sessions (Bldg 838) for all technicians on the following subjects:
    - space and caging requirements of laboratory animals (according to the revised Guide for the Care and Use of Laboratory Animals);
    - Herpes B virus in man;
    - zoonotic diseases of primates;

- proper methods of euthanasia in laboratory animals;
- automatic watering systems; and
- radiography – how it works.

A complete list of the programs and training materials used by the animal husbandry technicians is provided below:

| | |
|---|---|
| Purina Animal Care Course – a self study program | (12)[a] |
| Biotech Small Animal Series – small animal bleeding, oral dosing, handling, and restraint of lab animals | ( 7) |
| Good Laboratory Practices | ( 8) |
| Practical Training List | ( 5) |
| Advanced Practical Training List | ( 5) |
| Toxic Hazards Research Unit Animal Care | ( 6) |
| Laboratory Animal Medicine and Science Autotutorial Series | ( 7) |
| Dome Flight Training | ( 9) |
| Stark/McBride Technologist Correspondence Course | ( 7) |
| Medical Terminology | (12) |
| Toxic Hazards Research Unit Parapathologist Video Tapes | (10) |
| Laboratory Animal Technologist Video Tapes – preparation for AALAS certification (new course) | |

[a] The numbers in parentheses designate the technicians of our current group that have completed these courses.

To increase the capabilities, value, and versatility of the animal husbandry technicians, a program of cross-training in other areas has been implemented. All animal husbandry technicians have completed a form that identifies their skills and experience along with their areas of interest. The forms are reviewed when assistance is requested for research protocols. A monthly schedule is prepared to inform the technicians in advance of where they are needed.

### REFERENCE

MacEwen, J.D., E.H. Vernot. 1975. Toxic Hazards Research Unit Annual Technical Report. Report No. AMRL TR-75-57, Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

APPENDIX E

SUBMITTED TECHNICAL REPORTS AND JOURNAL PUBLICATIONS[a]

Technical Reports

Houston, W.F., R.S. Kutzman, and R.L. Carpenter. 1987. 1986 Toxic Hazards Research Unit Annual Report No. AAMRL-TR-87-020, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-87-2, Bethesda, MD: Naval Medical Research Institute.

Kinkead, E.R. and H.F. Leahy. 1987. Evaluation of the acute toxicity of selected groundwater contaminants. Report No. AAMRL-TR-87-021, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory.

Kinkead, E.R., C.L. Gaworski, A.P. D'Addario, and R.H. Bruner. 1987. The toxicological evaluation of polyalphaolefin hydraulic fluids. Report No. AAMRL-TR-87-038, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory, NMRI-87-18. Bethesda, MD: Naval Medical Research Institute.

Steele, V. 1987. Biological activity of chloropentafluorobenzene. Report No. AAMRL-TR-87-039, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory.

Kinkead, E.R., C.L. Gaworski, J.R. Horton, and T.R Boosinger. 1987. Chlorotrifluoroethylene oligomer: Evaluation of acute delayed neurotoxicity in hens, and study of absorption and metabolism in rats following oral, dermal, and inhalation exposure. Report No. AAMRL-TR-87-044, Wright-Patterson Air Force Bae, OH: Armstrong Aerospace Medical Research Laboratory.

Vinegar, A. 1987. Toxic effects of man-made mineral fibers with particular reference to ceramic fibers. Report No. AAMRL-TR-87-045, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-87-40, Bethesda, MD: Naval Medical Research Institute.

Kinkead, E.R., S.S. Henry, B.T. Culpepper, E.C. Kimmel, H.F. Leahy, and R.S. Kutzman. 1987. Evaluation of the acute toxicity of a synthetic polyalphaolefin-based hydraulic fluid. Report No. AAMRL-TR-87-053, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-87-55, Bethesda, MD: Naval Medical Research Institute.

Kinkead, E.R., D.L. Pollard, J.D. Collins, and E.J. Olajos. 1987. Evaluation of the acute toxicity of four compounds associated with the manufacture of o-ethyl-o'-(2-diisopropylaminoethyl)methyl-phosphonite. Report No. CRDEC-CR-87077. Aberdeen Proving Ground, MD: Chemical Research, Development, and Engineering Center.

Journal Publications

Carpenter, R.L., E.C. Kimmel, C.D. Flemming, and C.R. Doarn. 1987. Toxicant distribution in the Thomas domes. *Toxicology* 47(1,2):95-108.

PRESENTATIONS AT SCIENTIFIC MEETiNGS[a]

TOXICOLOGIC EFFECTS OF EXPERIMENTAL HYDRAULIC FLUIDS.   Henry, S.S., Kinkead, E.R., and Culpepper, S.T., Northrop Services, Inc., 101 Woodman Drive, Dayton, OH 45431; Porvaznik, M., and Bruner, R.H., Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, OH 45433.   Presented by S. S. Henry at the American Industrial Hygiene Association Conference, Montreal, CAN, 31 May - 5 June 1987.

Two categories of experimental hydraulic fluids were tested for a broad range of potential acute toxicities.  Two polyalphaolefin-based materials and four water-in-oil emulsions comprised the total of six test materials.   These fluids were developed for marine uses, as replacements for petroleum based products and as elements in high pressure submersible systems, respectively.  Oral, dermal and inhalation toxicity, eye and skin irritation, and skin sensitization tests were performed with each of the hydraulic fluids.  Oral, dermal and inhalation (four hour exposure) toxicity tests were single dose studies with 14-day post-exposure observation periods.  Male and female rats were used in the oral and inhalation studies, while dermal studies employed male and female rabbits.  Toxicity testing was initiated at an upper limit value, with $LC_{50}$ or $LD_{50}$ levels determined only when deaths occurred during limit testing.  Inhalation of the polyalphaolefin materials at 5 mg/l as aerosol (the limit test) proved fatal to 80% to 100% of test animals and three additional inhalation exposures at lower concentrations were performed with each test agent to determine $LC_{50}$ values of 1.6 and 1.2 mg/l for males and 1.4 and 0.8 mg/l for females.  The water-in-oil emulsions exhibited no toxicity at the maximum achievable aerosol concentrations of 0.1 to 0.2 mg/l.  Oral and dermal studies of all six hydraulic fluids did not demonstrate toxicity at the limit test values of 5.0 g/kg and 2.0 g/kg, respectively.  Eye and skin irritation tests were single applications on rabbits followed by a 72-hour observation period.  The polyalphaolefin-based test materials produced no eye or skin irritation in any of the test animals.  However, the water-in-oil emulsions evoked mild reactions in both eyes and skin.  Mild conjunctival redness was observed in up to 78% of eyes tested, although almost all of this was resolved by 72 hours after instillation.  In skin irritation studies all four water-in-oil emulsions produced slight erythema (but no edema or necrosis) in 17% to 83% of the rabbits tested.  Sensitization potential was determined by dosing guinea pigs four times over a ten day period, with a challenge dose administered two weeks later.  One of the polyalphaolefin and two of the water-in-oil fluids showed mild to moderate sensitization potential, while the remaining three test materials produced no sensitization reactions.

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CURRENT TOXICOLOGY REVIEW OF BERYLLIUM. 1987. Parnell, M.J., Henningsen, J.G., AAMRL/TH Wright-Patterson Air Force Base, OH: Carpenter, R.L., Northrop Services, Inc. Presented at the JANNAF Safety and Environmental Protection Subcommittee Meeting by M.J. Parnell, USAF, Cleveland, OH, May 4-5.

Increased USAF research and development in the area of rocket propellants has stimulated renewed interest in various chemical components of propellant systems. The toxicology of beryllium (Be) and most specifically beryllium oxide (Be), the principle exhaust product of Be solid propellant rocket motors, was recently reviewed. Ingestion and inhalation are considered the primary routes of Be exposure for humans and animals. Oral exposure to environmentally relevant levels of Be compounds has not been shown to produce any significant human or animal toxicity. However, acute and chronic toxicologic sequelae are recognized in both animals and humans following inhalation of various forms of Be, including BeO. Acutely, the inhalation of aerosols with high concentration of soluble Be compounds produces inflammation of the respiratory tract, especially the lungs which leads to pulmonary edema. In addition, a few cases of acute pneumonitis following repeated human exposure to low levels of Be have been reported. It is hypothesized that these low-level cases represent immune mediated allergic reactions to Be. Berylliosis was first described in fluorescent lamp workers in the 1940's, and is the term used to describe the chronic immunologically mediated disease which presents clinically as a granulomatous hypersensitivity response in the lung. Berylliosis generally has a slow onset, leading to progressive pulmonary insufficiency, and is reported to occur anywhere from 1 to 25 years after exposure. The carcinogencity of Be in several laboratory animal species is well documented; however, the human cancer risk is not well established. Recent epidemologic studies which report a correlation between Be exposure and an increased risk for lung cancer have been criticized on numerous grounds. The temperatures at which BeO is produced is reported to have a direct effect on the resulting toxicity. The "low-fired" BeO, calcined at 500 degrees C, appears to be more toxic and carcinogenic in experimental animals than the "high-fired" variety, calcined at 1600 degrees C. It has been suggested that an increased solubility in biological fluids of the "low-fired" BeO, compared to the "high-fired" BeO may account for this difference in toxicity.

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# APPENDIX G

## INVITED PRESENTATIONS[a]

Conolly, R.B. 1986. Pharmacodynamic Modeling of Cytotoxicity, Tissue Replication, and Mutation Accumulation. Presented at the 5th Annual Spring Meeting of the Ohio Calley Chapter of the Society of Toxicology, Cincinnati, OH, May 16.

Conolly, R.B. 1986. Biologically-Based Computer Modeling of Chemical Carcinogenesis. Presented at the New York University Medical Center, Institute of Environmental Medicine, Sterling Forest, Tuxedo, NY, June 5.

Conolly, R.B. 1987. Computer Simulation of Chemical Carcinogenesis. Presented at the University of Cincinnati Kettering Laboratories, Cincinnati, OH, October 7.

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## 1987 TOXIC HAZARDS RESEARCH UNIT GUEST SPEAKERS[a]

| DATE | TITLE | PRESENTER | THRU HOST |
|------|-------|-----------|-----------|
| 26 Jan 87 | Novel Uses of Ellmans Reagent and Chemical Analysis | Dr. Austin Raabe | Dr. R. Carpenter |
| 05 Feb | The Effects of Repeated Inhalation of Sulfuric Acid Aerosol on Tracheo-bronchial Mucociliary Function and Respiratory Mechanics in Rats | Dr. Jeffery Gearhart | Dr. R. Conolly |
| 06 Feb | Placental Drug and Steroid Metabolism | Dr. Peter Thomford | Dr. R. Conolly |
| 09 Feb | The Effects of Perfluorodecanoic Acid (PFDA) on Thyroid Status in Rats | Dr. Marc VanRafelgram | Dr. R. Conolly |
| 12 Feb | Anthracene Bioconcentration in Complex Chemical Mixtures | Dr. Gregory Linder | Dr. R. Conolly |
| 09 Mar | Herpes Virus Infection in Non-Human Primates | Dr. Thomas Davies | Dr. R. Kutzman |
| 16 Jul | Pathology of Acute Organophosphate Toxicity and the Effect of Treatments | Dr. Henry Wall, Jr. | Dr. R. Kutzman |
| 11 Aug | Hydrocarbon Type Analysis of Steam Cracker Feeds – Streamlining Classical Organic Analytical Methods for Modern Use | Dr. Joseph Soroka | Dr. R. Carpenter |
| 10 Sep | Cholesterol Distribution in Human Serum Low Density Lipoproteins and in Model Membranes | Dr. Gregory Smutzer | Dr. R. Kutzman |
| 14 Sep | Is Cigarette Smoking Neurotoxic? | Mr. Steven Goden | Dr. R. Kutzman |

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